Universidade de Aveiro Departamento de Biologia
Ano 2017

JOANA RITA MONTEIRO FIGUEIREDO

TOXICITY OF NOVEL ANTI-FOULING NANOMATERIALS IN MARINE ORGANISMS

TOXICIDADE DE NANOMATERIAIS ANTI-INCRUSTANTES INOVADORES EM ORGANISMOS MARINHOS

Universidade de Aveiro Departamento de Biologia
Ano 2017

JOANA RITA MONTEIRO FIGUEIREDO

TOXICITY OF NOVEL ANTI-FOULING NANOMATERIALS IN MARINE ORGANISMS

TOXICIDADE DE NANOMATERIAIS ANTI-INCRUSTANTES INOVADORES EM ORGANISMOS MARINHOS

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica do Doutor Roberto Carlos Domingues Martins, Investigador em Pós-Doutoramento do Departamento de Biologia e Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro, e co-orientação da Professora Doutora Susana Patrícia Mendes Loureiro, Professora Auxiliar com Agregação do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro.

DECLARAÇÃO

Declaro que este relatório é integralmente da minha autoria, estando devidamente referenciadas as fontes e obras consultadas, bem como identificadas de modo claro as citações dessas obras. Não contém, por isso, qualquer tipo de plágio quer de textos publicados, qualquer que seja o meio dessa publicação, incluindo meios eletrónicos, quer de trabalhos académicos.

o júri

Presidente	Prof. Doutor Carlos Manuel Martins Santos Fonseca Professor Associado com Agregação, Departamento de Biologia e Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro.
Arguente	Prof. Doutor Carlos Miguez Barroso Professor Auxiliar, Departamento de Biologia e Centro de Estudos do Ambiente e do Mar (CESAM), Universidade de Aveiro, Portugal
Orientador	Doutor Roberto Carlos Domingues Martins Investigador em Pós-Doutoramento, Departamento de Biologia e Centro de Estudos do Ambiente e do Mar (CESAM), Universidade de Aveiro, Portugal
Co-orientador	Prof. Doutora Susana Patrícia Mendes Loureiro Professora Auxiliar com Agregação, Departamento de Biologia e Centro de Estudos do Ambiente e do Mar (CESAM), Universidade de Aveiro, Portugal

agradecimentos

Ao Roberto Martins e Susana Loureiro, meus orientadores, por todo o apoio e ajuda durante a realização deste trabalho e pela oportunidade que me proporcionaram. Agradeço ainda todo o conhecimento que me transmitiram.

Aos meus pais, por sempre me apoiarem a seguir aquilo que desejei e por me darem força para nunca desistir.

Ao Maksym, por sempre me acompanhar ao longo deste percurso e por me apoiar em todos os momentos, bons e menos bons. Obrigada por me ajudares a continuar. люблю тебе!

palavras-chave

Nanomateriais; Bioincrustação marinha; Teste de exposição; Avaliação de perigosidade; SSDs; PNEC; Biocidas anti-vegetativos; DCOIT; Prata

resumo

A bioincrustação é uma sucessão ecológica de comunidades incrustantes em superfícies submersas que tem extensos impactos ecológicos, ambientais e económicos em todo o mundo quando desenvolvida em estruturas artificiais. Para minimizar esse problema, os biocidas com propriedades anti-incrustantes são comummente utilizados em revestimentos protetores de estruturas submersas. Há algumas décadas atrás, os compostos organoestânicos eram amplamente utilizados como agentes anti-incrustantes eficazes, porém foram definitivamente banidos em 2008 devido a efeitos tóxicos e de biomagnificação reportados. Como consequência, foi desenvolvida uma nova geração de biocidas com menor toxicidade e persistência no meio ambiente em comparação com os compostos organoestânicos. Recentemente, um desses biocidas (DCOIT) foi encapsulado num nanomaterial manufaturado (nanocápsulas de sílica mesoporosas, SiNC-DCOIT), a fim de evitar a interação dos biocidas com os ingredientes dos revestimentos e controlar a sua taxa de libertação durante o início de vida das tintas convencionais, com benefícios ambientais e económicos. O presente estudo teve como objetivo avaliar os efeitos em diversas espécies marinhas do nanomaterial SiNC-DCOIT e de uma versão modificada deste, contendo dois biocidas (SiNC-DCOIT revestido com prata), e comparar a sua toxicidade com os componentes destes nanomateriais (SiNC vazias, DCOIT e Ag).

Os testes de ecotoxicidade foram realizados com onze espécies marinhas, incluindo bactérias (*Vibrio fischeri*), microalgas (*Isochrysis galbana*, *Nannochloropsis gaditana*, *Phaeodactylum tricornutum*), rotíferos (*Brachionus plicatilis*), bivalves (*Cerastoderma edule*, *Mytilus galloprovincialis*), poliquetas (*Hediste diversicolor*), crustáceos (*Artemia salina, Palaemon varians*) e equinodermes (*Paracentrotus lividus*), seguindo testes padrão (com algumas adaptações em alguns casos) ou com protocolos bem definidos. Foram determinados parâmetros agudos ou crónicos de curta duração dependendo da espécie testada e do teste adotado. Globalmente, os valores de $L/E/IC_{50}$ para SiNC-DCOIT, SiNC-Ag e SiNC-DCOIT-Ag foram superiores aos valores estimados para DCOIT e prata (dissolvidos em solução), com exceção de alguns grupos alvo envolvidos nos primeiros estádios de incrustação, provando assim que estes são agentes alternativos mais amigos do ambiente comparativamente aos biocidas livres. Os valores obtidos de $L/E/IC_{50}$ e NOEC para os compostos testados foram depois utilizados para derivar curvas de distribuições de sensibilidade de espécies, juntamente com dados da literatura. Os valores HC_5 e PNEC derivados dessas curvas mostraram que o perigo do DCOIT e da prata diminui quando encapsulados, destacando que estes nanomateriais inovadores parecem ser uma solução anti-incrustante promissora.

keywords

Nanomaterials; Marine biofouling; Exposure testing; Hazard assessment; SSDs; PNEC; anti-vegetative biocides; DCOIT; silver

abstract

Biofouling is an ecological succession of fouling communities in submerged surfaces that has extensive ecological, environmental and economic impacts worldwide when developed over man-made structures. In order to minimize this problem, biocides with anti-fouling properties are commonly used in protective coatings of submerged structures. Some decades ago, organotin compounds were used as effective anti-fouling agents, however they were completely banned in 2008 due to the toxic and biomagnification effects. As a consequence, a new generation of biocides were developed with lower toxicity and persistence in the environment when compared to organotin compounds. Recently, one of these biocides (DCOIT) was encapsulated in an engineered nanomaterial (silica mesoporous nanocapsules, SiNC-DCOIT) in order to prevent the interaction of biocides with coatings' ingredients and control their leaching rate during the early lifetime of conventional paints, with environmental and economic benefits. The present study aimed to assess the toxicity of SiNC-DCOIT and a modified version of the engineered nanomaterial including two biocides, the SiNC-DCOIT coated with silver, to marine species and compare its toxicity with the free counterparts (empty SiNC, DCOIT and Ag).

Ecotoxicity tests were carried out with eleven marine species, including bacteria (*Vibrio fischeri*), microalgae (*Isochrysis galbana*, *Nannochloropsis gaditana*, *Phaeodactylum tricornutum*), rotifers (*Brachionus plicatilis*), bivalves (*Cerastoderma edule*, *Mytilus galloprovincialis*), polychaetes (*Hediste diversicolor*), crustaceans (*Artemia salina, Palaemon varians*) and echinoderms (*Paracentrotus lividus*), following standard tests (with some adaptions in some cases). Acute or short-term chronic endpoints were used upon each species and adopted test. Globally, values of $L/E/IC_{50}$ for SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag were higher than the estimated values for DCOIT and silver (dissolved in solution), except for some target groups involved in the early fouling stages, proving that these alternative agents are more environmentally-friendly comparatively to free biocides. The obtained $L/E/IC_{50}$ and NOEC values from the tested compounds were then used to create species sensitivity distributions together with data from literature. The HC_5 and PNEC values derivated from these curves showed that the hazard of DCOIT and silver is reduced when encapsulated, highlighting these novel nanomaterials as a promising anti-fouling solution.

Index

List of Figures

Chapter I

Chapter II

Chapter III

List of Tables

Chapter I

Chapter II

Chapter III

Abbreviations

Listed alphabetically

Ag	Silver
$AgNO_3$	Silver nitrate
ANOVA	One-way analysis of variance
Diuron	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DCOIT	4,5-Dichloro-2-octyl-4-isothiazolin-3-one
CTAB	Cetyltrimethylammonium bromide
Cu-PT	Copper pyrithione
ENMs	Engineered nanomaterials
HC_5	5% hazard concentration
Irgarol	2-(tert-Butylamino)-4-(cyclopropylamino)-6-(methylthio)-s-triazine
LDH	Layered double hydroxides
LOEC	Lowest observed effect concentration
MBT	Mercaptobenzothiazole
NPs	Nanoparticles
NMs	Nanomaterials
NOEC	No observed effect concentration
SiNC	Silica nanocapsules
SiNC-DCOIT	Silica nanocapsules loaded with DCOIT
SiNC-Ag	Silica nanocapsules coated with silver
SiNC-DCOIT-Ag	Silica nanocapsules loaded with DCOIT and coated with silver
SSD	Species sensitivity distribution
TBT	Tributyltin
Zn-PT	Zinc pyrithione

Chapter I

General Introduction

1. General Introduction

1.1. Nanomaterials and Marine Environment

Coastal zones and transitional waters (like estuaries and coastal lagoons) are areas of high productivity, essential to the function of all ecosystems (Blasco et al., 2016), providing between 14 and 22 trillion dollars in goods and services per year (Costanza et al., 1997; Harley et al., 2006). Therefore, due to the huge ecological and socioeconomic relevance of these habitats it is of major importance to promote their healthiness and their suitable and sustainable exploitation.

These ecosystems have been strongly affected by a wide variety of factors, such as climate and habitat changes, invasive species, eutrophication and chemical contaminants (Blasco et al., 2016), for which the marine ecosystem is regarded as a major sink (Hassan, 2002). There are several groups of chemical compounds present in the marine environment, namely metals, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, surfactants, pharmaceuticals, daily-care products, pesticides, herbicides, dioxins, nanomaterials, among others (Maruya et al., 2014; Johnston et al., 2015; Blasco et al., 2016). They can occur naturally and/or anthropogenically, posing a potential risk to living organisms and, indirectly, to humans (e.g. ingestion of fish and shellfish, direct skin contact) (Blasco et al., 2016). Nanomaterials are amongst the most important emergent compounds due to the exponential increase of engineered nanomaterials (ENMs) available in the market and, simultaneously, the lack of enough information regarding their toxicity, fate and behavior on the environment (Kahru and Dubourguier, 2010; McIntyre, 2012; Vance et al., 2015). According to the European Commission, a nanomaterial can be defined as *"a natural, incidental or manufactured material containing particles in an unbound state or as an aggregate or agglomerate and where, for 50% or more of the particles ins the number size distribution, one or more external dimensions is in the size range 1 nm – 100 nm"* and/or their area/volume ratio must be greater than 60 m^2/cm^3 (Recommendation 2011/696 EC, EC, 2011). Natural nanomaterials are abundant as they derive from several natural (biological or geological) mechanisms. For example, ash released during volcanic eruptions, soil erosion, sea salt aerosols formed when water droplets produced by waves evaporate and go to the atmosphere, combustion products from forest fires, dust storms and photochemical reactions occurring in atmosphere are some of the natural sources of nanomaterials (Buzea et al., 2007; Bhatt and Tripathi 2011; Alnashiri, 2015; Kumar and Kumbhat, 2016). On the other hand, human activities can lead to unintentional (incidental

nanomaterials) or intentional (ENMs) release of nanomaterials. The first ones include unintentionally produced materials or as by-products of human activities (e.g. welding, casting, fabrication of chemicals, atmospheric emissions, solids/liquids generated in production facilities). ENMs enter the environment through the use and production of commercial products (e.g. personal care products, paints and clothing). Furthermore, in rare situations a large volume of nanomaterials can enter the environment due to spillage during their transportation (Biswas and Wu, 2005; Buzea et al., 2007; Bhatt and Tripathi 2011; Alnashiri, 2015). Moreover, nanomaterials can enter in coastal areas through direct discharges, wastewater effluents, coastal erosion, river runoff and atmospheric deposition. Their levels in the water column, where they can interact with pelagic species (e.g. fish), will gradually increase and then precipitate in the substrate, where they can interact with benthic species (e.g. polychaetes, bivalves) (Alnashiri, 2015).

The quantification of ENMs in the environment is challenging and commonly a technical issue due to the intrinsic characteristics of the ENMs and the detection limits of the available state-of-the-art equipment. Currently, almost all measurement instruments and methods available to quantify these materials in environmental samples are non-specific, therefore it is difficult to differentiate between natural and manufactured particles, as well as ionic or nano forms. Besides, the background levels of natural nanomaterials are unknown (David, 2013; Sun et al., 2014). For this reason, most studies report the estimation of nanomaterial concentrations in the environment based on mathematical models (Gottschalk et al., 2013). Production volumes are used to generate estimates on their release from products and their subsequent distribution in environment. The resulting predicted environmental concentrations (PEC) can then be used for risk assessment (Arvidsson et al., 2011; Gottschalk et al., 2013; Sun et al., 2014). Yet, most studies are focused in few applications of ENMs and few presented quantitative estimations of their environmental concentrations (Sun et al., 2014). The study of Sun et al. (2014) is an exception, dealing with several production and input sources of nano-ZnO, nano-Ag, nano-TiO_2, carbon nanotubes and fullerenes. This is not surprising because these ENMs are presented in many products, are used in large amounts and their properties are well studied. Therefore, measure or predict the concentration of nanomaterials that do not fit on these categories is, currently, extremely difficult (Sun et al., 2014).

Despite the technical challenges and lack of enough scientific knowledge on environmental behavior, fate and ecotoxicity, nanomaterials started to be incorporated into products. However, since the late 2000s a growing concern about the possible adverse effects of nanomaterials to the environment and human health has emerged and, subsequently, there has been a significant increase in the number of scientific

papers in this area (Hansen, 2009; Kahru and Dubourguier, 2010). Several adverse effects of nanomaterials have been reported, such as neurotoxic effects, DNA damage, production of reactive oxygen species (ROS), malformations, reproductive effects, bioaccumulation and lethality (Hansen, 2009; Maurer-Jones et al., 2013; Baker et al., 2014; Avelelas et al., 2017; Martins et al., 2017). In this sense, ecotoxicology, *"the science that integrates the study of the ecological and toxicological effects of chemical pollutants on populations, communities and ecosystems with the fate (transport, transformation and degradation) of these pollutants in the environment"* (Forbes and Forbes, 1994), has a fundamental role in understanding the nanomaterial inherent effects and further on protection of the environment and, indirectly, humans.

Although the recent exponential increase on nanomaterials knowledge, their environmental effects are still not enough studied and scarcely understood (Ray et al., 2009; Kahru and Dubourguier, 2010; McIntyre, 2012; Maurer-Jones et al., 2013). The lack of information is even greater for the marine environment since the great majority of the nanotoxicology research have been focused in freshwater species (e.g. *Daphnia magna*, *Lymnaea stagnalis*, *Carnorhabditis elegans, Pimephales promelas*) (e.g. Handy et al., 2008; Baker et al., 2014). This is largely due to several practical questions (e.g. laboratorial cultures availability, the existing standard protocols), as well as due to the behavior and transformation processes (such as dissolution, dispersion, aggregation and agglomeration) of nanomaterials in estuarine or marine water. In the aquatic environment, the particles tend to aggregate and the extent of this aggregation depends on the particle size, shape, concentration and surface charge, on the medium pH and ionic strength and on the presence of natural organic matter (Batley et al., 2013; Maurer-Jones et al., 2013). In seawater (high ionic strength medium), the salinity reduces the negativity of electrophoretic mobility and promotes aggregation (Batley et al., 2013). In turn, aggregation increases the particle size and reduces its surface area, increasing also their stability. ENMs size is one of the properties that is known to affect the rate of dissolution (smaller particles dissolve faster) in exposure media and determine how they can enter organisms (bioavailability), tissues and cells of organisms (bioaccessibility). Agglomeration is a similar property to aggregation, with the main difference being the strength that particles are held together. Agglomerated nanomaterials are constituted by particles held together by weak physical forces (Van der Waals attraction), being therefore easier to separate using a mechanical process (e.g. ultrasounds). On the other hand, aggregated nanomaterials are strongly bonded and therefore not possible to again being individualized (Sokolov et al., 2015). Dissolution, besides depending on the size of the particles, also depends on the surface area (the smaller the surface area, the smaller the dissolution) (Baker et al., 2014). In addition, dissolution is also an extremely

important property since several studies have shown that nanomaterials become more harmful to organisms in their dissolved form(s) (Batley et al., 2013; Maurer-Jones et al., 2013; Baker et al., 2014). Due to these issues, saltwater testing is more challenging, especially with more complex nanomaterials.

Meanwhile, the industry is continuously and rapidly developing and producing novel nanomaterials that reach the market and environment without any type of control in terms of understanding their effects in wildlife and humans, relying on their non-nanoforms safety datasheets. In 2006 more than 300 products containing nanomaterials were available on the market (McIntyre, 2012), a number that exponentially increased to 1814 in 2015 (Vance et al., 2015). Moreover, the unique set of physical and chemical properties of nanomaterials, such as an higher surface:volume ratio and higher reactivity (Maurer-Jones et al., 2013; Vajtai, 2013), allows their easy manipulation and application in several areas. The commercial applications of these materials are, thus, almost unlimited, contributing for a new revolution in the world of materials. Nanomaterials can be used in several industrial areas, like the cosmetic industry (e.g. titanium dioxide NPs and zinc oxide NPs in sunscreens and toothpaste, iron oxide NMs in lipsticks, alumina NMs and silver NPs in soaps, shampoos, deo roll-ons and detergents), in the automotive industry (e.g. fullerene nanotubes composites in tires), remediation of industrial effluents, clothing, food industries, pharmaceuticals, biosensors, among many others (Biswas and Wu, 2005; Ray et al., 2009; Kahru and Dubourguier, 2010; McIntyre, 2012; Vajtai, 2013). More recently, nanomaterials were proposed as a technological advance to prevent and minimize two big and unsolved problems in maritime industry: corrosion and biofouling (Tedim et al., 2010; Maia et al., 2012; Zheludkevich et al., 2012; Maia et al., 2015; Avelelas et al., 2017). Maia et al. (2015) and Avelelas et al. (2017) proposed the use of functionalized nanoclays and mesoporous silica nanocapsules with booster biocides to be used as addivitives for protective paints to improve their performance in preventing marine biofouling in a more environmentally friendly way (for further details of this technique consult pp. 18–19 (Nanotechnological-based methods)).

1.2. Marine biofouling

Marine biofouling is an ecological succession of fouling communities on submerged surfaces, both natural (e.g. rocks, wood) and man-made (e.g. piers, platforms, ship hulls, buoys) (Jacobson and Willingham, 2000; Yebra et al., 2004; Gama et al., 2009; Hellio and Yebra, 2009; Cao et al., 2010). Globally there are more than 4000 fouling marine species (Yebra et al., 2004), most of which inhabit shallow waters along the coast and ports where organisms seak for higher nutrient levels (WHOI, 1952). These organisms can be divided into two large groups – microfoulers (e.g. bacteria and diatoms) and macrofoulers (e.g. algae, barnacles, polychaetes, bryozoans and mussels) (WHOI, 1952; Hellio and Yebra, 2009), which participates in the fouling process along four steps (Fusetani and Clare, 2006; Yebra et al., 2004) (Figure 1.1).

The first one begins with the formation of a conditioning film, resulting from the accumulation of organic molecules (such as polysaccharides, proteins and proteoglycans) and possibly of inorganic compounds on the surfaces (Loeb and Neihof, 1975; Lewin, 1984; Yebra et al., 2004; Fusetani and Clare, 2006; Hellio and Yebra, 2009).

The second stage consists on the physical adhesion, followed by adsorption, of bacteria and unicellular diatoms to the surface (Abarzua and Jakubowski, 1995; Hellio and Yebra, 2009; Maia, 2015) forming, together with protozoa and rotifers, a microbial biofilm (Yebra et al., 2004; Fusetani and Clare, 2006). The adsorption of bacteria is a process mainly conducted by physical forces, such as Van der Waals forces, electrostatic interaction and Brownian motion (Lewin, 1984; Abarzua and Jakubowski, 1995).

The third stage consists on the maturation of the microbial biofilm to a more complex community that usually includes multicellular primary producers, grazers and decomposers. These secondary colonizers (e.g. barnacle cyprids, spores of macroalgae, fungi and protozoa) are attrachted by the roughness of the microbial colonies and the existence of adhesive exudates (e.g. extracellular polymer substances, EPS), such as proteins, polysaccharides, lipids and nucleic acids (Yebra et al., 2004; Fusetani and Clare, 2006; Cao et al., 2010).

Lastly, the fourth phase involves the colonization and growth of large marine invertebrates (e.g. barnacles, polychaetes and mussels) and macroalgae, the so-called macrofoulers, resulting in complex colonies with a high diversity of fouling organisms (Abarzua and Jakubowski, 1995; Yebra et al., 2004; Fusetani and Clare, 2006; Hellio and Yebra, 2009; Cao et al., 2010; Maia, 2015). These macroorganisms have general characteristics like rapid growth rates, fast metamorphosis, low substrate preference and

high adaptability to different environments which enable them to be efficient foulers (Yebra et al., 2004).

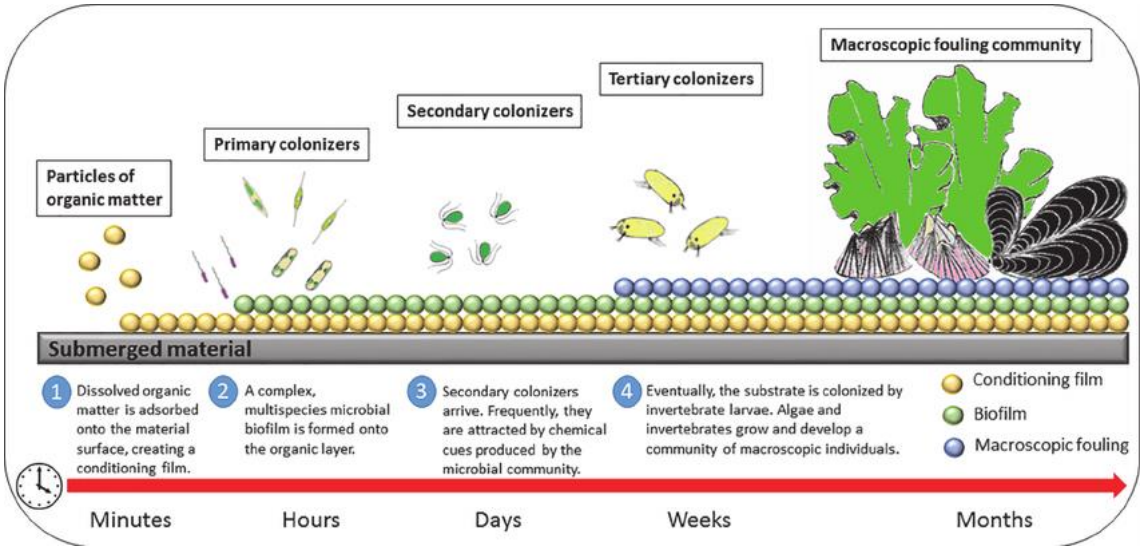


Figure 1.1 – Schematic representation of the biofouling process (Martín-Rodríguez et al., 2015).

1.3. Impacts of Marine Biofouling

Although biofouling is a natural process, when developed over man-made structures, it has extensive ecological, environmental and economic impacts worldwide. Some studies estimate that on a global scale at least 450 million dollars per year are spent on the prevention of biofouling, as this process reach losses of about 7 billion dollars per year worldwide (Gama et al., 2009). Others estimate that a highly efficient anti-fouling protection can save more than 150 billion dollars per year in 2020, excluding indirect costs resulting, for example, from delays in transport of goods and hull repairs (Hellio and Yebra, 2009). Despite the differences in these estimates, it is safe to state that biofouling must be prevented and minimized to avoid huge socio-economic losses.

In case of naval industry, the adverse effects caused by biological colonization are well known. Fouling leads to an increase of vessels' weight and makes the surface of the hulls irregular and rough, leading to an increase of friction resistance and consequent reduction in speed and loss of maneuverability. To offset this, increased fuel consumption is required, which leads to increased CO_2 emissions. Studies that include the fuel expenses estimate a total annual cost of approximately 3 trillion dollars due to fouling on vessels, since it can increase consumption by several hundred thousand liters (Jacobson and Willingham, 2000). It is estimated that in temperate waters fouling causes a 35-50% increase (depending on the type of vessel) in fuel consumption in a ship for a

6 month period at sea, and in tropical waters the increase may be much higher since incrustation develops faster (WHOI, 1952). In 2007 vessels released 870 million tons of CO_2, equivalent to 2.7% of the total emissions of this greenhouse gas in that year, with consequent impacts on climate change (Demirel et al., 2017). Fouling also causes an increase on ships' cleaning costs and on their time out of service, causing simultaneously huge economic losses (due to the inactivity of the vessel) and environmental impacts (large amount of toxic waste is generated during this process) (WHOI, 1952; Jacobson and Willingham, 2000; Gama et al., 2009; Hellio and Yebra, 2009; Cao et al., 2010).

In fixed structures (such as docks and oil rigs) fouling increases the mass of the installation and confers a distortion of the initial configuration of the structure. This process also leads to an increase in mass and reduction of buoyancy of floating devices (e.g. buoys, aquaculture cages) and to the reduction of the durability of marine pipelines (Gama et al., 2009; Hellio and Yebra, 2009). Associated with all these structures and vessels is the deterioration of their coating, promotion of the corrosion phenomena, discoloration and alteration of the electrical conductivity of materials (WHOI, 1952; Gama et al., 2009).

Biofouling also contributes to the dispersion of invasive/alien species through ballast water (planktonic, adult and resting stages) or vessel' hulls attachment (planktonic and adult stages) (Yebra et al., 2004; Gama et al., 2009; Hellio and Yebra, 2009). The negative impacts of introducing invasive species include possible competition (direct and/or indirect) and exclusion of native species, changes in food chain and habitat, reduction of biodiversity and economic losses (e.g. reduction of populations of commercial interest, costs for eradication and control measures) (Gama et al., 2009; Hellio and Yebra, 2009).

Taking into account the massive socio-economic and environmental consequences of biofouling on human-made structures, highly efficient anti-fouling techniques are especially required by industry and governments (Arai et al., 2009).

1.4. Conventional techniques for retard/prevent biofouling – Anti-fouling coatings

In the past, the application of coatings to the hull of vessels was already performed, being dated from Ancient Greece and even from earlier times. Pitch and copper were used to coat vessel' hulls by the early Phoenicians and Carthaginians. Wax, asphalt and tar were used by other ancient cultures. It is thought that these first coatings could serve for waterproofing, surface smoothing and protection against shipworms

(WHOI, 1952; Henwood, 1888; Hellio and Yebra, 2009). Lead coating was later used to coat hulls in Ancient Rome and Greece (WHOI, 1952; Readman, 2005). Despite the low efficacy of lead in the protection against incrustation, this coating was probably efficient in the protection of wooden hulls, becoming one of the most widely used methods and adopted by countries such as Spain, France and England (WHOI, 1952; Yebra et al., 2004).

Later, the use of lead was discontinued because it was found that it causes corrosion on the iron components of vessels, in addition to the discovery of higher anti-fouling efficacy coatings, e.g. based on copper (Yebra et al., 2004). Despite its effectiveness, only in the 19th century was clarified, through the study of the corrosion process of copper, that this metal was dissolving in seawater which prevented biofouling (WHOI, 1952). However, after the introduction of ships with iron hulls the use of copper coatings almost disappeared since, like lead, they caused corrosion when in direct contact with iron (Henwood, 1888; Readman, 2005; Hellio and Yebra, 2009). This triggered the development of new highly-performant anti-fouling coatings (Readman, 2005; Hellio and Yebra, 2009). In substitution of copper, the paints were prepared by adding compounds such as arsenic, zinc, nickel and mercury oxide to resin binders. However, it was found that these paints became ineffective in just over a year, despite the high efficacy against foulers in the 1st year (WHOI, 1952; Yebra et al., 2004; Readman, 2005). Vessels would then often have to be dry-docked, scraped and repainted, as well as the fixed structures, being these solutions very costly and not always effective (Gama et al., 2009).

In the late 1950s, organotin compounds, especially TBT (tributyltin), were found to be effective liposoluble pesticides (as they can penetrate the cell membranes), with long duration and with the additional advantage of not causing corrosion in hulls, and thus rapidly replaced the previous toxic additives in anti-fouling paints (Yebra et al., 2004; Readman, 2005; Gama et al., 2009; Hellio and Yebra, 2009). Due to its high performance (5 times higher comparing with Cu-based coatings), the application of TBT paints rapidly expanded, representing roughly 70% of the anti-fouling top-coatings worldwide (Readman 2005; Zhou, 2015).

Although it appeared to be a promising solution for biofouling, with the beginning of the large-scale use of TBT paints in the early 1970s the first studies of the deleterious effects of this compound on non-target species also began to appear (Evans et al., 1995; Arai et al., 2009; Gama et al., 2009; Cao et al., 2010). Two of the most well documented cases of its impact on non-target species are malformations in oyster shells (*Crassostrea gigas*) and imposex in gastropods (e.g. *Nassarius reticulatus*, *Nucella lapillus*) (Barroso et al., 2000, 2002; Axiak et al., 2003; Arai et al., 2009; Galante-Oliveira et al., 2010;

Higuera-Ruiz and Elorza, 2011). Remarkably, these effects can be observed at very low exposure concentrations, namely 20 and 1 ng/L, respectively (Thain, 1986; Evans et al., 1995; Evans, 1999). Other negative effects included the accumulation of TBT in organisms and effects on the immunological defense of fish and other species (Evans, 1999; Arai et al., 2009; Zhou et al., 2015). This way, due to its high toxicity and bioaccumulation, and with a view to environmental protection, the use of TBT-containing paints was completely banned on 17 September 2008[1] (IMO, 2001).

Currently, anti-fouling coatings can have biocides or not (Maia, 2015). However, the application of anti-fouling coatings containing biocides is the most widely-used method to protect submerged structures by the modern maritime industries due to their higher efficacy and cost-effectivness (Zhou, 2015). Moreover, there are three different ways of releasing the biocide from the paint, namely based on the soluble matrix, insoluble matrix and self-polishing paint. The technique based on the soluble matrix dissolve in time and do not assure protection for more than 12-15 months, needing constant maintenance. Therefore, nowadays it is not very used. Paints with insoluble matrix keep the matrix intact while the biocide itself dissolves slowly, leaving an exhausted matrix of paint that must be removed before the surface is re-coated. Lastly, the self-polishing paint is "self-polishing", with gradual release of the biocide and copolymers (Yebra et al., 2004; Almeida et al., 2007; Maia, 2015).

The development of these coatings involves the selection of the metal (e.g. lead, zinc, copper) or organic (e.g. pyrithione, isothiazolones) active compound, the matrix/binder (e.g. resin, silyl or metal acrylates), the pigments (e.g. iron oxide, zinc oxide), the extenders/fillers (e.g. barium sulphate, calcium carbonate) and the solvents (e.g. xylene, butyl acetate) (Candries, 2000; Hellio and Yebra, 2009). Briefly, the matrix/binder determines most of the physico-chemical properties of the paint, provides a continuous film that contribute for the adhesion to the surface and to the general resistance of the coating to the environment (Candries, 2000; Hellio and Yebra, 2009). The wide variety of pigments contribute to the color, opacity and anti- fouling and/or corrosive properties of the paint. Extenders are normally colorless and used to adjust the total volume of pigment to the required level. Solvents dissolve the paint binder and reduce its viscosity to a level that facilitates its application by the selected method (e.g. brush, spray) (Candries, 2000; Hellio and Yebra, 2009). Generally, additives that may have various functions, such as corrosion inhibition, UV absorption, dispersion, modification of electrical properties, etc., are also used (Hellio and Yebra, 2009).

[1] http://www.imo.org/en/About/Conventions/ListOfConventions/Pages/International-Convention-on-the-Control-of-Harmful-Anti-fouling-Systems-on-Ships-(AFS).aspx

Looking at the example of biocides, these are commonly incorporated or bound to the binder corresponding to the active ingredients that are released from the paint to prevent biofouling. The efficacy of the biocide differs on its concentration and time of exposure. For that reason, biocides' leaching rate from the paint is of extreme importance, since it will determine the amount of biocide released and must be maintained high enough to prevent fouling during the lifetime of the paint (Gama et al., 2009; Maia, 2015).

Due to the complete ban of TBT in 2008, it was urgent to develop new strategies to tackle biofouling, as efficient as organotin compounds but much more eco-friendly than these compounds. For this, the ideal anti-fouling agent must prevent the growth and fixation of hundreds of species without causing adverse effects on the marine environment, thus it may have rapid degradation and partitioning in environment resulting in limited bioavailability for non-target organisms, low toxicity to non-target species at concentrations present in the environment and minimum bioaccumulation (Jacobson and Willingham, 2000). So, a new generation of biocides without organotin was developed, with lower toxicity, bioaccumulation and persistence in the environment. These compounds are organic booster biocides (e.g. Copper and Zinc Pyrithione, Irgarol 1051, Diuron, Zineb, Ziran, DCOIT, etc.) containing elements of nitrogen, halogen, sulfur and boron, which may include heterocyclic amines, aromatic halides, carbamates, phenols, albyl amines and phosphorus compounds (Thomas, 2001; Dafford et al., 2011; Zhou, 2015). These compounds are being used isolated or together with Cu-based compounds to maximize the anti-fouling efficacy of the coatings. Within this group of organic biocides, DCOIT (4,5-Dichloro-2-octyl-4-isothiazolin-3-one), also commercially known as Sea-Nine 211N or Kathon 287T, was found to be a very promising solution as it is a broad-spectrum anti-fouling agent with low environmental risk, since it rapidly degrades in seawater and sediment (Jacobson and Willingham, 2000; Dafforn et al., 2011). DCOIT causes oxidative stress inducing the production of free radicals followed by necrosis and disruption of metabolic pathways by inhibiting dehydrogenase enzymes, blocking the activity of the enzyme glutathione reductase, consuming cellular glutathione reserves and inhibiting the ATP synthesis (Williams, 2007; Wendt et al., 2016). It also affects several enzymes in the Krebs' cycle of microbial organisms (Hellio and Yebra, 2009).

This biocide is extremely toxic to a wide variety of aquatic organisms, however, its rapid degradation in seawater significantly reduces its concentration below toxic levels. Its main degradation process is biological, and hydrolysis and photolysis do not play a significant role on its degradation in the environment (Willingham and Jacobson, 1996; Jacobson and Willingham, 2000; Yebra et al., 2004). Several studies have shown that its half-life time in natural saltwater (containing microorganisms) is lower than 24

hours, and its degradation by photolysis and hydrolysis takes 9-12.5 days and 13.4 days, respectively (Shade et al., 1993; Willingham and Jacobson, 1996; Thomas, 2001). In samples of water with a low number of bacteria (< 1000 bacteria/mL) the half-life time is higher (76-187h) (Shade et al., 1993). This is not relevant for natural conditions since it does not represent the biological activity of seawater, however, it may be important in laboratory context since sterilized water may be used to perform toxicological tests and, therefore, prolonged half-life times may be present. On the other hand, DCOIT and the by-products resulting from its decomposition rapidly and strongly bind to the sediment and, once bound, become immobile, reducing its bioavailability (Jacobson et al., 1993). In addition, the by-products resulting from its decomposition are open ring structures and their toxicity is usually reduced by 4-5 orders of magnitude (Jacobson et al., 1993; Jacobson and Willingham, 2000). These characteristics also confer low risk of accumulation in the food chain (Jacobson and Willingham, 2000). Although DCOIT presents severe short-term toxicity for many target and non-target organisms (Table 1.1), like other booster biocides (Table 1.2), most studies show that no long-term toxicological effects are observed (Shade et al., 1993; Willingham and Jacobson, 1996; Jacobson and Willingham, 2000).

Besides booster biocides, there are other compounds, like silver nitrate, now being applied for anti-fouling purposes. Silver is known to have antibacterial activity against a range of Gram-positive (e.g. *Bacillus*, *Enterococcus*, *Listeria*) and Gram-negative (e.g. *Vibrio*, *Escherichia*, *Pseudomonas*) bacteria (Clement and Jarrett, 1994; Wijnhoven et al., 2009) and against some fungi (Marambio-Jones and Hoek, 2010). This anti-microbial activity relies on the diffusion of Ag^+ ions from the substrate material (Lelieveld et al., 2016). Silver is already widely used in a range of applications (such as domestic water filters and silver-coated ceramic filters) to reduce the level of biofilm growth (Nguyen et al., 2012; Fewtrell, 2014). Moreover, although primarily known by its action against microorganisms, several studies demonstrated that silver (Ag^+) has deleterious effects on other organisms at very low concentrations (Table 1.3), including species that can be part of the biofouling process (namely some microalgae, diatoms, bivalves and crustaceans). Therefore, silver nitrate has high potential to be applied in anti-fouling compounds, despite it was not yet submitted for approval in the list of active substances of the category PT21 (anti-fouling products) under the European Biocidal Products Regulation (BPR) (EU Regulation 528/2012).

Table 1.1 – Toxicity data ($L/E/IC_{50}$) for marine organisms exposed to DCOIT retrieved from an extensive literature review.

Organism	Species	Endpoint	Value (mg/L)	Reference
Bacteria	*Vibrio fischeri*	30 min EC_{50}	0.003	Fernández-Alba et al. (2002)
Cyanobacteria	*Synechococcus* sp.	72 h EC_{50}	0.001	Devilla et al. (2005)
Microbial	*Periphyton community*	72 h EC_{50}	0.026	Arrhenius et al. (2006)
Microalgae	*Emiliania huxleyi*	72 h EC_{50}	0.0004	Devilla et al. (2005)
Diatoms	*Halamphora coffeiformis*	LC_{50}	0.003	Jacobson and Willingham (2000)
	Skeletonema costatum	96 h EC_{50}	0.014	Shade et al. (1993)
	Skeletonema costatum	96 h EC_{50}	0.020	Willingham and Jacobson (1996)
	Skeletonema costatum	96 h EC_{50}	0.018	EPA (1992)
	Skeletonema costatum	96 h EC_{50}	0.026	Wendt (2013)
Macroalgae	*Ulva lactuca*	72 h EC_{50}	0.023	Wendt et al. (2013)
	Ulva intestinalis	120 h EC_{50}	0.002	Jacobson and Willingham (2000)
	Hormosira banksii germination	48 h EC_{50}	0.340	Myers et al. (2006)
	Hormosira banksii rhizoid growth	48 h EC_{50}	0.430	Myers et al. (2006)
	Fucus serratus zygotes	24 h EC_{50}	0.019	Braithwaite and Fletcher (2005)
Bivalves	*Mytilus edulis* adult	48 h EC_{50}	0.851	EPA (1992)
	Mytilus edulis embryo	48 h EC_{50}	0.411	DCOIT assessment report (2014)
	Mytilus edulis embryo	48 h EC_{50}	0.011	Bellas (2006)
	Mytilus edulis embryo	48 h EC_{50}	0.003	EPA (1992)
	Crassostrea virginica embryo	48 h EC_{50}	0.009	EPA (1992)
	Crassostrea virginica embryo	48 h LC_{50}	0.024	Willingham and Jacobson (1996)
	Crassostrea virginica embryo	48 h EC_{50}	0.012	DCOIT assessment report (2014)
	Magallana gigas eggs	24 h LC_{50}	0.017	Tsunemasa and Okamura (2011)
Crustaceans	*Acartia tonsa*	48 h LC_{50}	0.016	Wendt et al. (2016)
	Acartia tonsa	72 h EC_{50}	0.038	Hjorth et al. (2006)
	Tigriopus japonicus	24 h LC_{50}	0.030	Yamada (2007)
	Americamysis bahia	96 h LC_{50}	0.005	Shade et al. (1993)
	Penaeus japonicus	96 h LC_{50}	0.013	Yamada (2007)
	Penaeus aztecus	96 h LC_{50}	0.012	Shade et al. (1993)
	Penaeus aztecus	96 h LC_{50}	0.016	Heitmuller (1977)
	Penaeus aztecus	96 h LC_{50}	0.027	EPA (1992)
	Amphibalanus amphitrite larvae	24 h LC_{50}	0.340	Jacobson and Willingham (2000)
	Amphibalanus amphitrite	24 h EC_{50}	0.220	Willemsen et al. (1998)
	Leptuca pugilator	96 h LC_{50}	1.31	Shade et al. (1993)
	Leptuca pugilator	96 h LC_{50}	1.70	EPA (1992)
Ascidiacea	*Ciona intestinalis* embryo	24 h EC_{50}	0.105	Belas (2006)
	Ciona intestinalis larval settlement	24 h EC_{50}	0.043	Belas (2006)
Echinoderms	*Paracentrotus lividus* 4-arm larvae	48 h EC_{50}	0.019	Bellas (2007)
	Paracentrotus lividus 4-arm larvae	48 h EC_{50}	0.012	Bellas (2006)
	Paracentrotus lividus larval growth	48 h EC_{50}	0.025	Bellas (2006)
	Paracentrotus lividus larval growth	48 h EC_{50}	0.021	Bellas (2007)
	Glyptocidaris crenularis 4-arm larvae	53 h EC_{50}	0.001	Xu et al. (2010)
Fish	*Cyprinodon variegatus variegatus*	96 h LC_{50}	0.023	EPA (1992)
	Cyprinodon variegatus variegatus	96 h LC_{50}	0.017	Shade et al. (1993)
	Pagrus major	96 h LC_{50}	0.005	Kawashima (1997)
	Takifugu rubripes	96 h LC_{50}	0.006	DCOIT assessment report (2014)

Table 1.2 – Toxicity data (L/E/IC_{50}) for marine organisms exposed to different booster biocides retrieved from an extensive literature review.

Contaminant	Organism	Species	Endpoint	Value (mg/L)	Reference
Diuron	Cyanobacteria	*Synechococcus* sp.	96h EC_{50}	0.110	Bao et al. (2011)
	Cyanobacteria	*Synechococcus* sp.	72h EC_{50}	0.001	Devilla et al. (2005)
	Microalgae	*Dunaliella tertiolecta*	24h EC_{50}	0.035	McFeters et al. (1983)
	Microalgae	*Emiliania huxleyi*	72h EC_{50}	0.002	Devilla et al. (2005)
	Microalgae	*Raphidocelis subcapitata*	72h EC_{50}	0.045	Mezcua et al. (2002)
	Diatoms	*Cylindrotheca closterium*	72h IC_{50}	0.017	Stauber et al. (2008)
	Diatoms	*Entomoneis punctulata*	72h IC_{50}	0.024	Stauber et al. (2008)
	Diatoms	*Skeletonema costatum*	96h EC_{50}	0.006	Bao et al. (2011)
	Diatoms	*Thalassiosira pseudonana*	96h EC_{50}	0.004	Bao et al. (2011)
	Dinoflagellate	*Pyrocystis lunula*	24h EC_{50}	43.0	Bao et al. (2011)
	Dinoflagellate	*Pyrocystis lunula*	24h EC_{50}	19.0	Stauber et al. (2008)
	Macroalgae	*Hormosira banksii*	48h EC_{50}	6.75	Myers et al. (2006)
	Coralline algae	*Neogoniolithon fosliei*	24h IC_{50}	0.009	Negri et al. (2011)
	Bivalves	*Crassostrea virginica*	96h EC_{50}	4.80	EPA (1992)
	Polychaetes	*Hydroides elegans*	48h LC_{50}	16.0	Bao et al. (2011)
	Crustaceans	*Trigiopus japonicus*	96h LC_{50}	11.0	Bao et al. (2011)
	Crustaceans	*Artemia salina*	24h LC_{50}	12.0	Koutsaftis and Aoyama (2007)
	Crustaceans	*Amphibalanus amphitrite*	24h LC_{50}	21.0	Bao et al. (2011)
	Crustaceans	*Palaemon serratus*	24h LC_{50}	3.01	Bellas et al. (2005)
	Crustaceans	*Americamysis bahia*	96h LC_{50}	1.10	EPA (1992)
	Echinoderms	*Paracentrotus lividus*	48h EC_{50}	5.60	Bellas et al. (2005)
	Coral	*Acropora valida*	24h LC_{50}	4.80	Bao et al. (2011)
	Fish	*Cyprinodon variegatus*	96h LC_{50}	0.890	EPA (1992)
	Fish	*Oryzias melastigma*	96h LC_{50}	7.80	Bao et al. (2011)
Irgarol	Cyanobacteria	*Synechococcus* sp.	96h EC_{50}	0.023	Bao et al. (2011)
	Microalgae	*Chroococcus minor*	96h EC_{50}	0.008	Zhang et al. (2008)
	Microalgae	*Dunaliella tertiolecta*	24h EC_{50}	0.001	Gatidou and Thomaidis (2007)
	Diatoms	*Skeletonema costatum*	96h EC_{50}	0.001	Bao et al. (2011)
	Diatoms	*Thalassiosira pseudonana*	96h EC_{50}	0.0004	Bao et al. (2011)
	Macroalgae	*Ulva intestinalis*	72h EC_{50}	0.003	Scarlett et al. (1997)
	Macroalgae	*Eisenia bicyclis*	96h EC_{50}	0.006	Okamura et al. (2000)
	Macroalgae	*Hormosira banksii*	48h EC_{50}	3.54	Seery et al. (2006)
	Coralline algae	*Pyropia yezoensis*	96h EC_{50}	0.0001	Okamura et al. (2000)
	Polychaetes	*Hydroides elegans*	48h LC_{50}	2.60	Bao et al. (2011)
	Crustaceans	*Trigiopus japonicus*	96h LC_{50}	2.40	Bao et al. (2011)
	Crustaceans	*Artemia salina*	24h LC_{50}	1.60	Panagoula et al. (2002)
	Crustaceans	*Amphibalanus amphitrite*	24h LC_{50}	2.20	Bao et al. (2011)
	Crustaceans	*Elasmopus rapax*	96h LC_{50}	1.00	Bao et al. (2011)
	Crustaceans	*Palaemonetes pugio*	96h LC_{50}	2.46	Key et al. (2008)
	Crustaceans	*Americamysis bahia*	96h LC_{50}	0.400	EPA (1992)
	Gastropods	*Ilyanassa obsoleta*	96h LC_{50}	3.73	Finnegan et al. (2008)
	Fish	*Oryzias melastigma*	96h LC_{50}	1.00	Bao et al. (2011)
	Fish	*Menidia beryllina*	96h LC_{50}	1.58	EPA (1992)
	Fish	*Cyprinodon variegatus*	96h LC_{50}	3.50	EPA (1992)
Zn-PT	Cyanobacteria	*Synechococcus* sp.	96h EC_{50}	0.022	Bao et al. (2011)
	Microalgae	*Tetraselmis chuii*	96h IC_{50}	0.280	Avelelas et al. (2017)
	Diatoms	*Thalassiosira pseudonana*	96h EC_{50}	0.0005	Bao et al. (2011)
	Diatoms	*Thalassiosira pseudonana*	96h EC_{50}	0.002	Bao et al. (2008)

	Diatoms	*Skeletonema costatum*	96h EC_{50}	0.002	Bao et al. (2011)
	Diatoms	*Phaeodactylum tricornutum*	96h EC_{50}	0.010	Avelelas et al. (2017)
	Dinoflagellate	*Pyrocystis lunula*	24h EC_{50}	0.0004	Bao et al. (2011)
	Bivalves	*Crassostrea virginica*	96h EC_{50}	0.022	EPA (1992)
	Bivalves	*Mytilus edulis*	96h LC_{50}	0.211	Avelelas et al. (2017)
	Polychaetes	*Hydroides elegans*	48h LC_{50}	0.008	Bao et al. (2011)
	Crustaceans	*Tigriopus japonicus*	96h LC_{50}	0.170	Bao et al. (2011)
	Crustaceans	*Artemia salina*	24h LC_{50}	3.17	Koutsaftis and Aoyama (2007)
	Crustaceans	*Elasmopus rapax*	96h LC_{50}	0.029	Bao et al. (2008)
	Crustaceans	*Heptacarpus futilirostris*	96h LC_{50}	120	Mochida et al. (2006)
	Crustaceans	*Amphibalanus amphitrite*	24h LC_{50}	0.210	Bao et al. (2011)
	Crustaceans	*Americamysis bahia*	96h LC_{50}	0.005	EPA (1992)
	Cnidarians	*Aiptasia sp.*	96h LC_{50}	0.410	Bao et al. (2011)
	Coral	*Acropora valida*	24h LC_{50}	0.180	Bao et al. (2011)
	Fish	*Oryzias melastigma*	96h LC_{50}	0.043	Bao et al. (2011)
	Fish	*Pagrus major*	96h LC_{50}	98.2	Mochida et al. (2006)
	Fish	*Cyprinodon variegatus*	96h LC_{50}	0.400	EPA (1992)
Cu-PT	Cyanobacteria	*Synechococcus* sp.	96h EC_{50}	0.022	Bao et al. (2011)
	Microalgae	*Tetraselmis chuii*	96h IC_{50}	0.300	Avelelas et al. (2017)
	Diatoms	*Thalassiosira pseudonana*	96h EC_{50}	0.0007	Bao et al. (2011)
	Diatoms	*Phaeodactylum tricornutum*	96h EC_{50}	0.010	Avelelas et al. (2017)
	Dinoflagellate	*Pyrocystis lunula*	24h EC_{50}	0.023	Bao et al. (2011)
	Polychaetes	*Hydroides elegans*	48h LC_{50}	0.006	Bao et al. (2011)
	Crustaceans	*Tigriopus japonicus*	96h LC_{50}	0.030	Bao et al. (2011)
	Crustaceans	*Tigriopus japonicus*	96h LC_{50}	0.074	Bao et al. (2008)
	Crustaceans	*Artemia salina*	24h LC_{50}	0.830	Koutsaftis and Aoyama (2007)
	Crustaceans	*Elasmopus rapax*	96h LC_{50}	0.011	Bao et al. (2011)
	Crustaceans	*Heptacarpus futilirostris*	96h LC_{50}	2.50	Mochida et al. (2006)
	Crustaceans	*Amphibalanus amphitrite*	24h LC_{50}	0.063	Bao et al. (2011)
	Coral	*Acropora valida*	24h LC_{50}	0.028	Bao et al. (2011)
	Fish	*Oryzias melastigma*	96h LC_{50}	0.0082	Bao et al. (2011)
	Fish	*Pagrus major*	96h LC_{50}	9.30	Mochida et al. (2006)

Table 1.3 – Toxicity data ($L/E/IC_{50}$) for marine organisms exposed to silver retrieved from an extensive literature review.

Organism	Species	Endpoint	Value (mg/L)	Reference
Bacteria	*Vibrio fischeri*	30 min EC_{50}	0.600	Georgantzopoulou et al. (2012)
	Vibrio fischeri	15 min EC_{50}	0.464	Rosen et al. (2008)
Cyanobacteria	*Synechococcus* sp.	72 h EC_{50}	0.097	Burchardt et al. (2012)
Microalgae	*Isochrysis galbana*	48 h LC_{50}	0.081	Wilson and Freeburg (1980)
Diatoms	*Thalassiosira pseudonana*	72 h EC_{50}	0.129	Burchardt et al. (2012)
	Ditylum brightwellii	120 h EC_{50}	0.008	Canterford and Canterford (1980)
Dinoflagellate	*Ceratocorys horrida*	24 h EC_{50}	0.008	Rosen et al. (2008)
	Pyrocystis pseudonoctiluca	24 h EC_{50}	0.038	Rosen et al. (2008)
	Lingulodinium polyedrum	24 h EC_{50}	0.006	Rosen et al. (2008)
	Gymnodinium splendens	48 h LC_{50}	0.021	Wilson and Freeburg (1980)
Bivalves	*Mytilus edulis*	48 h EC_{50}	0.014	Martin et al. (1981)
	Crassostrea virginica embryo	48 h LC_{50}	0.006	Calabrese et al. (1977)
	Magallana gigas embryo	48 h EC_{50}	0.022	Martin et al. (1981)
	Magallana gigas larvae	48 h EC_{50}	0.019	Dinnel et al. (1983)
	Argopecten irradians	96 h LC_{50}	0.033	Nelson et al. (1976)
	Mercenaria mercenaria	42-48 h LC_{50}	0.021	Calabrese and Nelson (1974)

Polychaetes	*Hediste diversicolor*	96 h LC_{50}	0.650	Mouneyrac et al. (2003)
	Neanthes arenaceodentata	96 h EC_{50}	0.132	Pesch and Hoofman (1983)
	Neanthes arenaceodentata	96 h EC_{50}	0.254	Pesch and Hoofman (1983)
	Neanthes arenaceodentata	96 h EC_{50}	0.119	Pesch and Hoofman (1983)
	Neanthes arenaceodentata	96 h EC_{50}	0.260	Pesch and Hoofman (1983)
	Neanthes arenaceodentata	96 h EC_{50}	0.145	Pesch and Hoofman (1983)
	Neanthes arenaceodentata	96 h EC_{50}	0.151	Pesch and Hoofman (1983)
Crustaceans	*Acartia tonsa*	48 h EC_{50}	0.163	Pedroso et al. (2007)
	Acartia tonsa	48 h LC_{50}	0.043	Hook and Fisher (2001)
	Acartia clausi	96 h LC_{50}	0.043	Lussier and Cardin (1985)
	Acartia hudsonica	48 h LC_{50}	0.043	Hook and Fisher (2001)
	Tisbe battagliai	24 h LC_{50}	0.167	Macken et al. (2012)
	Tisbe battagliai	48 h LC_{50}	0.091	Macken et al. (2012)
	Tigriopus brevicornis	96 h LC_{50}	0.095	Barka et al. (2001)
	Tigriopus brevicornis	96 h LC_{50}	0.129	Forget et al. (1995)
	Tigriopus brevicornis	96 h LC_{50}	0.121	Menasria and Pavillon (1994)
	Tigriopus brevicornis	96 h LC_{50}	0.088	Forget et al. (1995)
	Tigriopus brevicornis	96 h LC_{50}	0.159	Menasria and Pavillon (1994)
	Americamysis bahia	96 h LC_{50}	0.178	Schimmel (1981)
	Americamysis bahia	96 h LC_{50}	0.117	Schimmel (1981)
	Americamysis bahia	96 h LC_{50}	0.264	Schimmel (1981)
	Americamysis bahia	96 h LC_{50}	0.251	Schimmel (1981)
	Americamysis bahia	96 h LC_{50}	0.248	Schimmel (1981)
	Americamysis bahia	96 h LC_{50}	0.203	Schimmel (1981)
	Americamysis bahia	96 h LC_{50}	0.250	Nacci et al. (1986)
	Americamysis bahia	96 h LC_{50}	0.065	Schimmel (1981)
	Metacarcinus magister	96 h LC_{50}	0.055	Martin et al. (1981)
Gastropoda	*Nassarius reticulatus* larvae	96 h EC_{50}	0.044	Zahra Khodaparast (2015)
Echinoderms	*Strongylocentrotus purpuratus*	40 min EC_{50}	0.115	Bay et al. (1993)
	Strongylocentrotus droebachiensis	96 h EC_{50}	0.100	Dinnel et al. (1982)
	Arbacia punctulata	96 h EC_{50}	0.040	Ward et al. (2006)
Fish	*Menidia menidia*	96 h LC_{50}	0.110	Nacci et al. (1986)
	Pseudopleuronectes americanus larvae	96 h LC_{50}	0.296	Cardin (1981)
	Pseudopleuronectes americanus larvae	96 h LC_{50}	0.503	Lussier and Cardin (1985)
	Paralichthys dentatus larvae	96 h LC_{50}	0.005	Cardin (1980)
	Paralichthys dentatus eggs	96 h LC_{50}	0.016	Lussier and Cardin (1985)
	Oligocottus maculosus	96 h LC_{50}	0.664	Shaw et al. (1998)
	Oligocottus maculosus	96 h LC_{50}	0.636	Shaw et al. (1996)
	Oligocottus maculosus	96 h LC_{50}	0.331	Shaw et al. (1998)
	Fundulus heteroclitus heteroclitus	96 h LC_{50}	0.040	Bielmyer et al. (2008)
	Fundulus heteroclitus heteroclitus	96 h LC_{50}	2.70	Dorfman (1977)
	Cyprinodon variegatus variegatus	96 h LC_{50}	1.08	Schimmel (1981)
	Cyprinodon variegatus variegatus	96 h LC_{50}	1.58	Schimmel (1981)
	Cyprinodon variegatus variegatus	96 h LC_{50}	0.640	Schimmel (1981)
	Cyprinodon variegatus variegatus	96 h LC_{50}	1.18	Schimmel (1981)
	Cyprinodon variegatus variegatus	96 h LC_{50}	0.441	Schimmel (1981)
	Apeltes quadracus	96 h LC_{50}	0.547	Lussier and Cardin (1985)
	Paraphys vetulus	96 h EC_{50}	0.800	Dinnel et al. (1983)
	Cymatogaster aggregata	96 h EC_{50}	0.356	Dinnel et al. (1989)
	Oncorhyunchus mykiss	96 h LC_{50}	0.402	Fergunson and Hogstar (1998)
	Oncorhyunchus kisutch	96 h EC_{50}	0.488	Dinnel et al. (1989)
	Squalus acanthus	96 h LC_{50}	0.100	Boeck et al. (2001)

1.5. Emergent techniques for retard/prevent biofouling

1.5.1. Biomolecules

Some organisms have the capacity to inhibit the growth of their competitors through physical, chemical or ecological mechanisms, individually or in combination (Bers et al., 2006). Recently, researchers have explored these chemical defense mechanisms, namely the segregation of enzymes or metabolites with anti-fouling properties, and attempted to extract high concentrations of these biodegradable and low toxicity components for use in environmentally-friendly anti-fouling coatings (Bers et al., 2006; Fusetani and Clare, 2006; Hellio and Yebra, 2009; Cao et al., 2010; Zhou, 2015). It has already been found that organisms such as blue algae (Abarzua et al., 1999), sponges (Fusetani and Clare, 2006; Mol et al., 2009), fungi (Xiong et al., 2009) and bacteria (Burgess et al., 2003; Fusetani and Clare, 2006) can produce effective components for the prevention of biofouling. As an example, quorum-sensing inhibitors are molecules (e.g. lactones) produced by bacteria, which have been regarded as promising anti-biofilm agents (Yang et al., 2016). The functions of enzymes may be the degradation of adhesives used for fixation of organisms, biofilm matrix disruption, interference with intercellular communication and catalyzing the release of compounds with anti-fouling properties (Kristensen et al., 2008; Cao et al., 2010). Some examples of enzymes with anti-fouling capacity are peroxidases, oxidoreductases, transferases, lyases, isomerases, ligases and hydrolases (Kristensen et al., 2008).

One of the challenges of biologically based anti-fouling compounds is finding a balance between the effectiveness and the lifetime of the coating. The fact that seawater temperature ranges from -2 to 36 °C can affect the stability and catalytic capacity of enzymes and interfere with the lifespan of the coating. Moreover, it is necessary to conceive an appropriate matrix to contain enzymes, since it is essential that enzymes contact with water for catalytic activity and they also need to have structural mobility. However, a problem associated with the need for structural mobility is the solubilization of enzymes for which the encapsulation/immobilization have been proposed as a solution for their fast leaching (Kristensen et al., 2008; Cao et al., 2010). Moreover, enzymes must also meet four requirements: (a) have a broad-spectrum action; (b) retain its activity when mixed with the remaining components of the coating; (c) not decrease coating performance; (d) have a long-term stability in the dry coating and after submerging the coated surface (Olsen et al., 2007).

1.5.2. Biomimetic strategies

Biomimetization is defined as the study of the structure and function of biological systems and processes as models for the manufacture of materials and machines (Hellio and Yebra, 2009; Salta et al., 2010). One of the strategies studied for anti-fouling coatings is mimic the grooved scales of sharks to produce surfaces based on their skin. It was found that the presence of these placoid scales (spiny projections that cover the skin of sharks and that are nearly parallel to their longitudinal body axis) reduces the hydrodynamic drag in 5-10%. These scales also provide protection against ectoparasites, thereby having anti-fouling properties with potential for use in coatings (Hellio and Yebra, 2009; Magin et al., 2010; Salta et al., 2010). It has also been found that the presence of microscopic pores and nano-ridges surrounded by an enzymatically secreted gel on the skin of the pilot whale gives it resistance to microorganisms, since this gel denatures proteins and carbohydrates (Baum et al., 2002; Yebra et al., 2004).

The mussel *Mytilus edulis* also seems to be able to produce bioactive compounds with anti-fouling properties in its shell, since this organism can remain free of fouling organisms if it has an intact periostracum (i.e. the outer layer of the shell, composed exclusively of organic material). The ripple-like microtopography of the shell of this species also serves as a defense mechanism against fixing organisms (Bers et al., 2006; Magin et al., 2010; Salta et al., 2010).

One of the major limitations and challenges of using biomimetization for the development of anti-fouling surfaces is the scalability (e.g scale up). The differences between natural systems and artificial structures and vessels in terms of dimension and scale are significant and difficult to overcome. This requires a large volume of any natural anti-fouling product that can be incorporated into a coating (Salta et al., 2010). Therefore, it is a technique that is not often use or find available.

1.5.3. Electrochemical methods

Electrolysis of seawater is another method for preventing biofouling. With this process it is possible to produce hypochlorous acid, ozone, bromine or hydrogen peroxide that will spread through the hulls of vessels and, due to their strong oxidizing capacity, can remove fouling organisms (Yebra et al., 2004). One of the problems associated with this method is that in some cases the coatings are not very efficient due to a large drop in tension along the surface that will increase the corrosion problems of the hulls (Cao et al., 2010).

Methods based on the direct electron transfer between an electrode and microbial cells (microcrosmic electrochemical methods), have also been proposed as means of causing electrochemical oxidation of the intercellular substances. Yet this method is expensive and its efficacy as not yet been assessed (Cao et al., 2010).

1.5.4. Fouling-release coatings

Fouling-release or non-stick coatings are biocide-free coatings that provide a low-friction, ultra-smooth surface that difficult organisms' adhesion. They are regarded as self-cleaning coatings since fouling organisms can be easily removed mechanically (e.g. with a brush or a water jet) or hydrodynamically during navigation. The first-generation of these coatings was based on silicones and the second-generation on fluoropolymers (e.g. polytetrafluorethylene (PTFE) like TEFLON®) (Anderson et al., 2004; Nendza, 2007; Hellio and Yebra, 2009; Lejars et al., 2012). More recently a hydrogel-silicone-based alternative was proposed as a third-generation coating and has been regarded as more efficient (Thorlaksen, 2010; Lejars et al., 2012). However, these coatings are still expensive and susceptible to mechanical damage and they have poor adhesion to the substrate as well as fouling-release properties at low speed (it is required a speed above 30 knots to remove fouling organisms hydrodynamically, especially biofilms) (Yebra et al., 2004; Lejars et al., 2012). It was even observed that the increase in fuel consumption and CO_2 emissions due to the microbial biofilm that these coatings do not effectively prevent could have higher environmental impact than the higher toxicity related to the use of biocides (Hellio and Yebra, 2009). Other studies propose combining this technique with biocides at low concentrations, increasing the efficacy but producing more environmentally-friendly systems comparing with the traditional biocide-based coatings (Yebra et al., 2004; Lejars et al., 2012).

1.5.5. Physical methods

It has been found that, to some extent, barnacles and mussels may be removed by vibration method (Branscomb and Rittschof, 1984). However, the enormous energy consumption associated with this method has not yet been overcome (Cao et al., 2010). Other studies have also evaluated magnetic fields, radioactive coatings and ultraviolet radiation for anti-fouling purposes, but the application of these methods is not considered very convenient and practical (Yebra et al., 2004).

1.5.6. Nanotechnological-based methods

Due to the difficulties and challenges of the emerging technologies mentioned above, it is likely that in the near future they still cannot be able to overcome the efficacy of anti-fouling coatings based on biocides as they remain the most durable, resistant, easy to apply, low maintenance and cost-effective. However, the legislation of these compounds is increasingly becoming stricter in order to set the protection of human, animal and environmental health as crucial, so it becomes imperative to investigate new ways of making these typical coatings more environmentally friendly, reducing their toxicity but maintaining their efficacy. Herewith, studies that utilize the encapsulation of biocides have emerged in order to control their release and, thus, reduce the toxicity of the coatings and promote environmental protection. Two examples are the encapsulation of biocides in latex nanocapsules (Zang et al., 2007) and in chitosan/xanthan gum micro-containers with a core-shell structure (Borodina et al., 2014). This safer-by-design solution was also successfully applied in protective coatings with anti-corrosion purposes through the encapsulation of the corrosion inhibitors in ENMs, namely in silica mesoporous nanocapsules (SiNC) (Maia et al., 2012) and in layered double hydroxides (LDH) (Tedim et al., 2010; Zheludkevich et al. 2012; Martins et al., 2017). SiNC are characterized by having spherical morphology and diameter size ranging between 100 and 150 nm (Maia et al., 2015). These nanocontainers are prepared through an oil-in-water microemulsion containing the surfactant CTAB (cetyl trimethylammonium bromide), that serves as emulsion stabilizer and template for the condensation of TEOS (tetraethyl orthosilicate), followed by the hydrolysis and condensation of TEOS at the microemulsion's interface (Maia et al., 2015; Avelelas et al., 2017). These ENMs have been regarded as low toxic compounds. The toxicity of SiNC was recently assessed in the microalgae *Tetraselmis chuii* (96 h IC_{50} = 22.7 mg SiNC/L), the diatom *Phaeodactylum tricornutum* (96 h IC_{50} = 3.67 mg SiNC/L) (Avelelas et al., 2017), the bryozoan *Bugula neritina* (24 h EC_{50} = 0.1 mg SiNC/L) and the bivalve *Brachidontes pharaonis* (72 h LC_{50} = 13.2 mg SiNC/L) (Gutner-Hoch et al., submitted). This demonstrates that the most sensitive species to SiNC is the bryzoan *B. neritina*, which participates in the biofouling process.

More recently, a research group from the University of Aveiro used nanotechnology-based processes to develop and produce, at laboratory scale, a new functional coating using engineered nanomaterials. They encapsulated DCOIT in silica mesoporous nanocapsules (SiNC) in order to prevent the interaction of the active compounds with paint formulations, preventing their inactivation and allowing their controlled release under the action of a predefined stimulus (Maia, 2015). The

encapsulation of DCOIT in SiNC is prepared in an oil-in-water solution by adding ammonia solution and DCOIT diluted in ethyl eter to an aqueous solution containing SiNC and CTAB. The final loading content of DCOIT in SiNC is 18.3%. SiNC loaded with DCOIT have larger size than the empty nanocapsules (approximately 300 nm) (Maia et al., 2015). DCOIT is released from SiNC over time under predefined stimulus like pH, temperature and concentration of NaCl, mainly by diffusion (Maia et al., 2015).

Currently, the Portuguese company Smallmatek, Lda. is making the scale-up of this new material and exploring the potential of its production at the industrial scale, having simultaneously developed other solutions, namely the encapsulation of zinc and copper pyrithiones in SiNC and LDH (SiNC-ZnPT, SiNC-CuPT, LDH-ZnPT and LDH-CuPT). Avelelas et al. (2017) showed that the immobilization of these two biocides (Zn-PT and Cu-PT) into nanostructured nanomaterials seems to be a promising eco-friendly strategy without compromising the anti-fouling efficacy comparing with the free biocides.

In order to improve and increase the anti-fouling capacity of SiNC-DCOIT, this company has also developed a new form of this material. These silica nanocapsules loaded with DCOIT were coated with a silver nitrate film, forming a new engineered nanomaterial containing two biocides (SiNC-DCOIT-Ag). Silver is a bactericidal agent and acts against the formation of microbial biofilms (Wijnhoven et al., 2009; Nguyen et al., 2012; Fewtrell, 2014), one of the first and key steps of biofouling process. Although the biocidal capacity of SiNC-DCOIT has been experimentally demonstrated (Maia, 2015), the possible toxic effects of these new compounds that may be used in anti-fouling coatings have not yet been assessed, corresponding to the main goal of this study.

1.6. Legislation in Europe

The European Union has established two major regulations regarding chemical products, namely REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) and CLP (Classification, Labeling and Packaging of substances and mixtures), that came into force in 1 June 2007 and are applicable to all sectors of the industry that work with chemicals and the entire supply chain. This legislation makes companies responsible for the safety of the chemicals they place on the market. REACH is "*a regulation adopted to improve the protection of human health and the environment from the risks that can be posed by chemicals, while enhancing the competitiveness of the EU chemicals industry. It also promotes alternative methods for the hazard assessment of substances in order to reduce the number of tests on animal*s" and CLP "*ensures that the hazards presented by chemicals are clearly communicated to workers and consumers in the European Union through classification and labelling of chemicals*"[1].

Regarding anti-fouling paints, the European Union has established its first registration system of anti-fouling paints under the Biocidal Products Directive (BPD) that entered into force in 2000. Posteriorly, this regulation was repealed and replaced by the Regulation 528/2012 (EU), which entered into force on September 1, 2013 (EU, 2012). This new regulation simplifies the requirements for the approval and authorization of active substances and products. It also leads to the reduction of animal tests through sharing of data and the encouragement of a more flexible approach of tests. This regulation is the first to include the new definition of the European Commission for nanomaterials, submitted on October 18, 2011[2].

The registration system established by European Union requires that anti-fouling products (e.g. paints) and their active ingredients (e.g. biocides) be authorized, demanding producers to clarify the safety of the products through risk assessment methodologies. For products to be authorized, a dossier containing the required dataset must be submitted to one of the Member States of the European Union (EU, 2012). More specifically, for the active ingredients is required to provide general information on the substance, efficacy against target organisms, exposure assessment, evaluation of effects on human health and hazard identification. It is also necessary to demonstrate the use of the active substance in real products, so the dossier must also contain information for a product containing that substance (EU, 2012). The information for human health effects includes toxicokinetic and metabolic data, carcinogenicity, irritation, corrosivity, genotoxicity, neurotoxicity, etc. The information for environmental effects includes data on destination, distribution, degradation and accumulation in the environment and effects on aquatic and terrestrial organisms (EU, 2012). Through the

data acquired in these topics it will be possible to determine the predicted environmental concentration (PEC) and the predicted no-effect concentration (PNEC) and characterize the risk of the substance (Arai et al., 2009). All these requirements are based on a previous study of the fate and destiny of chemicals to accurately test relevant exposure scenarios.

The submitted proposals are evaluated and, if approved, the products are placed in an attached list depending on their level of risk. In this case, if the PEC/PNEC ratio is smaller than 1, the application will be approved and the product/substance will be placed on the list. If the ratio is greater than 1, the risk/benefit analysis is performed. In case there is no alternative product or the elimination of the product from the market brings significant disadvantages, it may still be approved but is subject to conditions (e.g. continuous monitoring of the environment) (Arai et al., 2009; EU, 2012). Thus, this system provides a list of permitted substances and aims to harmonize the norms on the availability on the market and use of biocidal products in the countries of the European Union, while ensuring a high level of protection for human, animal and environmental health (EU, 2012).

In Portugal, biocidal products are regulated not only by European legislation but also by its transposition to national legislation. The current legislation includes: Decreto-Lei n.º 121/2002, changed by Decreto-Lei n.º 112/2010 on the legal regime for placing in the market biocidal products; Decreto Lei n.º 82/2003 (transposes Directive 1999/45/EC), on the approximation of the laws, regulations and administrative provisions of the Member States relating to the classification, packaging and labeling of dangerous preparations, adapted to technical progress by Directive 2001/60/EC and, in the case of dangerous preparations, Directive 2001/58/EC; Portaria n.º 702/2006 which establishes the fees to be paid by applicants for authorization to place biocidal products in the market); Regulation (EC) n.º 1451/2007.

[1] https://echa.europa.eu/regulations

[2] http://www.sgs.pt/pt-PT/Consumer-Goods-Retail/Cosmetics-Personal-Care-and-Household/Detergents-and-Household-Care/Regulations/EU-Biocides-Regulation.aspx

1.7. Use of Species Sensitivity Distribuitions (SSDs) to assess ecological hazard and risks

The potential hazard of toxic compounds to ecosystems has led scientists to look for ways to estimate environmentally safe concentrations and methods to assess ecological hazard and risks. One of the major difficulties is to estimate the effects on various species and ecosystems because organisms have a wide diversity of traits, such as life history, taxonomy, behavior, physiology, morphology and geographical distribution (Posthuma et al., 2002). These differences cause different species to respond differently to a given concentration of a chemical compound, i.e., different species have different exposure routes and sensitivities (Forbes and Callow, 2002; Posthuma et al., 2002). This led, almost simultaneously in United States and Europe, to the idea of using a species sensitivity distribution (SSD) curve for the assessment of ecological hazard and derivation of environmental quality criteria (Posthuma et al., 2002; Maltby et al., 2005).

SSDs are then one of the methods to approach the variation in sensitivity of species to an exposure to toxics through a statistical distribution function, without trying to explain the cause of this variation (Forbes and Callow, 2002; Posthuma et al., 2002; Maltby, 2005; Silva et al., 2014). These curves are used to estimate the hazardous concentration (HC) which affects a certain proportion of a set of species (*p*%), being that the HC_5 is generally the one estimated (i.e. hazardous concentration to 5% of species) (Aldenberg and Jaworska, 2000; Posthuma et al., 2002; Maltby et al., 2005; Garner et al., 2015). This means that the compound is considered to be hazardous if the probability of selecting a species from the set with LC_{50} value, for instance, smaller than the estimated concentration is equal to 5% (Aldenberg and Jaworska, 2000). The HC_5 value can then be used to establish environmental quality objectives, i.e., if the concentration of the compound in the environment is below this value, 95% of the considered species are protected (Aldenberg and Jaworska, 2000; Maltby et al., 2005; Garner et al., 2015). Moreover, from the 5^{th} percentile (HC_5) of the SSD it is possible to calculate the predicted no-effect concentration (PNEC) after the application of an assessment factor between 1 and 5 (Poshuma et al., 2002; van Vlaardingen and Verbruggen, 2007; Silva et al., 2014).

The main assumption in the application of SSDs is that the toxicity data of the chosen species is representative and allows to extrapolate to a community or ecosystem level. The more data available for several species, the greater the accuracy of these curves in predicting the effects on ecosystems (Garner et al., 2015). To assess the hazard in aquatic environment several authors suggest that at least data for 8-10 species should be used. If less data is used there will be greater variability in the model

production and the created estimates for the evaluated effect levels can be unreliable (Wheeler et al., 2002).

This approach has the advantage of providing an overview of the likelihood of toxicity of the compounds for the selected species and offers the possibility to aggregate data from different studies. Moreover, if the curves are constructed with relevant species of diverse trophic levels and phyla it is possible to have a closer idea of the potential impact of the chemical in the environment (Silva et al., 2014). On the other hand, although this assessment aims to protect communities and ecosystems, it is usually done using a toxicity dataset of several species evaluated individually (Posthuma et al., 2002; Maltby et al., 2005; Garner et al., 2015). Thus, some of the criticisms to this model are not evaluating any interaction between species, not taking into account the ecosystem functioning and also the bioavailability of the toxicant (Aldenberg and Jaworska, 2000).

On the other hand, some studies report that it is preferable to use data from chronic toxicity tests for the construction of SSDs because organisms are often environmentally exposed to chemicals in a chronic way (Kooijman, 1987; Aldenberg and Jawoska, 2000; Wheeler et al., 2002). Although chronic endpoints (ex: growth, development, reproduction) are ecologically more relevant, the use of acute toxicity data has several key advantages. Firstly, acute tests require less laboratory demand due to the shorter exposure time. For example, feeding does not normally take place and it is not necessary to renew the medium during the test period, also making acute tests more cost-effective than chronic tests. In chronic tests it is already necessary to successfully keep organisms in the laboratory for a long period of time, so it is only possible to carry them out with a limited number of species (Posthuma et al., 2002). Secondly, unlike chronic tests, there are many standardized acute toxicity tests, guaranteeing uniformity, reliability and reproducibility and with the additional advantage of making it easier for decision makers to accept test results. Besides, for most chemical compounds the available chronic toxicity data is insufficient to create reliable SSDs, but generally there are sufficient acute toxicity data and from species belonging to all major taxonomic groups (Kooijman, 1987; Posthuma et al., 2002; Maltby et al., 2005). In addition, acute toxicity data refer to a limited number of responses and time scales (e.g. lethal concentrations at 96 hours), usually easier to interpret, while chronic toxicity data correspond to a wide range of responses and test durations, introducing additional variability in curves (Maltby et al., 2005). Last, but not least, the risks associated with biocides (especially DCOIT) (Dafforn et al., 2011) are generally of short-term because of their rapid degradation in the environment and therefore their effect will be more accurately assessed through data from acute tests.

1.8. Thesis aims

Accordingly to the above, the general aims of the present study are:

- To evaluate the exposure effects of several novel anti-fouling nanomaterials (SiNC, SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag) in several marine species from lower trophic levels to higher trophic levels (Decomposers – Primary producers – Primary and secundary consumers);
- To assess if the encapsulation of the biocides in silica nanocapsules reduces their toxicity to non-target species and maintain efficacy towards fouler species;
- To assess the environmental hazard of biocides, nano-strucutred biocides and empty nanomaterial and understand which are the most sensitive taxa.

Specific objectives:

- To assess the acute and short-term chronic toxicity in eleven relevant marine species and to calculate NOEC, LOEC and L/E/IC_{50} values;
- To compare the obtained results with literature for the same compounds;
- To compare the toxicity of DCOIT and silver with other state-of-the art biocides;
- To understand if the possible toxicity of “empty” silica nanocapsules is associated with the inherent surfactant CTAB (used in its production);
- To assess the hazard of the tested compounds by deriving and comparing PNEC values obtained with different statistical (deterministic and SSDs) and functional (species and phylum) approaches;
- To compare the obtained HC_5 and PNEC values with values for the same or other chemical compounds from the literature.

1.9. Relevancy of the study

Biofouling has massive ecological and socioeconomic impacts worldwide, which can bring losses of millions of dollars. Over the years various techniques and compounds have been applied to prevent this natural process. However, many of them have been discontinued due to the low anti-fouling efficacy, high costs and maintenance or toxicity against non-target species even at very low concentrations (like TBT). More recently, new environmentally-friendly techniques have been developed and tested, some of them without the use of chemical compounds (e.g. biomimetization, biomolecules, vibration methods), but they still can not overcome the advantages of biocides (especially

applicability, technical issues and costs). Therefore, novel techniques to develop and produce less toxic biocides are being employed based on nanotechnology. This has led to the encapsulation of biocides in manufactured nanomaterials (e.g. layered double hydroxides (LDH) and silica nanocapsules (SiNC)) with the aim of controlling their release to the environment. Two examples of the use of this technique are the encapsulation of the biocide DCOIT in silica nanocapsules (SiNC-DCOIT) and a modified version of this nanomaterial using a silver nitrate coating (SiNC-DCOIT-Ag).

Although encapsulation appears to be a promising technique for producing more environmentally-friendly anti-fouling compounds (Maia et al., 2015; Avelelas et al., 2017; Martins et al., 2017), there is few information on the effects of these new compounds on target and, especially, non-target species. Thus, this study can help companies producing this type of nanomaterials to decide which are the most environmentally friendly approaches, by providing information on their toxicity in a set of species of different trophic levels, serving as reference for future work. Moreover, it also allows to complement the existing literature for DCOIT and silver.

1.10. Thesis organization

The present work was organized in four chapters:

- The first chapter is the “General Introduction” to the themes of nanomaterials and marine environment, marine biofouling and its ecological and socio-economic impacts, conventional and emergent techniques to prevent biofouling, legislation of biocidal products in Europe and the use of Species Sensitivity Distributions to assess ecological hazard and risks;
- The second chapter entitled “Toxicity of innovative anti-fouling nano-based solutions in marine species”, settles the exposure effects of SiNC-DCOIT, SiNC-Ag, SiNC-DCOIT-Ag and their free counterparts (SiNC, DCOIT and Ag^{+}) on eleven marine species from different trophic levels, accordingly to different protocols depending on the test organism;
- The third chapter entitled “Use of species sensitivity distribution curves to assess the hazard of new anti-fouling nano-based solutions in marine species” presents a full hazard assessment of the tested compounds, where SSDs have been constructed, with data from this study and literature, in order to calculate HC_5 values and PNEC values for SiNC, DCOIT, Ag^{+}, SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag.

- The fourth chapter entitled “General discussion and final considerations” presents a holistic discussion of the main findings as well as the main conclusions that arose from the present study. This chapter also includes a reflection on possible future works.

1.11. References

Abarzua, S., and Jakubowski, S. (1995). Biotechnological investigation for the prevention of biofouling. I. Biological and biochemical principles for the prevention of biofouling. M*arine Ecology Progress Series*, 123, 301–312. doi:10.3354/meps123301.

Abarzua, S., Jakubowski, S., Eckert, S., and Fuchs, P. (1999). Biotechnological Investigation for the Prevention of Marine Biofouling II. Blue-Green Algae as Potential Producers of Biogenic Agents for the Growth Inhibition of Microfouling Organisms. *Botanica Marina*, 42(5), 459–465. doi:10.1515/bot.1999.053.

Aldenberg, T., and Jaworska, J. (2000). Uncertainty of the Hazardous Concentration and Fraction Affected for Normal Species Sensitivity Distributions. *Ecotoxicology and Environmental Safety*, 46(1), 1–18. doi:10.1006/eesa.1999.1869.

Almeida, E., Diamantino, T., and Sousa, O. (2007). Marine paints: The particular case of antifouling paints. *Progress in Organic Coatings*, 59(1), 2–20. doi: 10.1016/j.porgcoat.2007.01.017.

Alnashiri, H. (2015). The ecotoxicology of different forms of copper (nano, micro and salt) in marine mussels. PhD (Doctor of Philosophy) thesis, School of Life Sciences, Heriot-Watt University, Scotland.

Anderson, C., Atlar, M., Callow, M., Candries, M., Milne, A. and Townsin, R. (2004). The development of foul-release coatings for seagoing vessels. *Journal of Marine Design and Operations*, B4, 11–23.

Arai, T., Harino, H., Ohji, M., and Langston, W. (2009). Ecotoxicology of Antifouling Biocides. Japan: Springer. ISBN: 978-4-431-85708-2.

Arrhenius, A., Backhaus, T., Grönvall, F., Junghans, M., Scholze, M., and Blanck, H. (2006). Effects of Three Antifouling Agents on Algal Communities and Algal Reproduction: Mixture Toxicity Studies with TBT, Irgarol, and Sea-Nine. Archives of Environmental Contamination and *Toxicology*, 50(3), 335–345. doi:10.1007/s00244-005-1057-9.

Arvidsson, R., Molander, S., Sandén, B., and Hassellöv, M. (2011). Challenges in Exposure Modeling of Nanoparticles in Aquatic Environments. *Human and Ecological Risk Assessment: An International Journal*, 17(1), 245–262. doi:10.1080/10807039.2011.538639

Avelelas, F., Martins, R., Oliveira, T., Maia, F., Malheiro, E., Soares, A., Loureiro, S., and Tedim, J. (2017). Efficacy and Ecotoxicity of Novel Anti-Fouling Nanomaterials in Target and Non-Target Marine Species. *Marine Biotechnology*, 19(2), 164–174. doi:10.1007/s10126-017-9740-1.

Axiak, V., Micallef, D., Muscat, J., Vella, A., and Mintoff, B. (2003). Imposex as a biomonitoring tool for marine pollution by tributyltin: some further observations. *Environment International*, 28(8), 743–749. doi:10.1016/s0160-4120(02)00119-8.

Baker, T., Tyler, C., and Galloway, T. (2014). Impacts of metal and metal oxide nanoparticles on marine organisms. *Environmental Pollution*, 186, 257–271. doi:10.1016/j.envpol.2013.11.014.

Bao, V., Leung, K., Qiu, J., and Lam, M. (2011). Acute toxicities of five commonly used antifouling booster biocides to selected subtropical and cosmopolitan marine species. Marine pollution bulletin, 62(5), 1147–1151. doi:10.1016/j.marpolbul.2011.02.041.

Barka, S., Pavillon, J., and Amiard, J. (2001). Influence of different essential and non-essential metals on MTLP levels in the Copepod *Tigriopus brevicornis*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 128(4), 479–493. doi:10.1016/s1532-0456(00)00198-8.

Barroso, C., Moreira, M., and Gibbs, P. (2000) Comparison of imposex and intersex development in four prosobranch species for TBT monitoring of a Southern Europe estuarine system (Ria de Aveiro, NW Portugal). *Marine Ecology Progress Series*, 201, 221–232. doi:10.3354/meps201221.

Barroso, C., Moreira, M., and Bebianno, M. (2002). Imposex, female sterility and organotin contamination of the prosobranch *Nassarius reticulatus* from the Portuguese coast. *Marine Ecology Progress Series*, 230, 127–135. doi:10.3354/meps230127.

Batley, G., Kirby, J., and Mclaughlin, M. (2013). Fate and Risks of Nanomaterials in Aquatic and Terrestrial Environments. *Accounts of Chemical Research*, 46(3), 854–862. doi:10.1021/ar2003368.

Baum, C., Meyer, W., Stelzer, R., Fleischer, L. and Siebers, D. (2002). Average nanorough skin surface of the pilot whale (*Globicephala melas*, Delphinidae): considerations on the self-cleaning abilities based on nanoroughness. *Marine Biology*, 140(3), 653–657. doi:10.1007/s00227-001-0710-8.

Bay, S., Burgess, R., and Nacci, D. (1993) Status and applications of echinoid (phylum Echinodermata) toxicity test methods *in* Landis, W., and Hughes, J. (1993) Environmental toxicology and risk assessment. Philadelphia: American Society for Testing and Materials.

Bellas, J., Beiras, R., Marino-Balsa, J., and Fernández, N. (2005). Toxicity of Organic Compounds to Marine Invertebrate Embryos and Larvae: A Comparison Between the Sea Urchin Embryogenesis Bioassay and Alternative Test Species. *Ecotoxicology*, 14(3), 337–353. doi:10.1007/s10646-004-6370-y.

Bellas, J. (2006). Comparative toxicity of alternative antifouling biocides on embryos and larvae of marine invertebrates. *Science of The Total Environment*, 367(2–3), 573–585. doi:10.1016/j.scitotenv.2006.01.028.

Bellas, J. (2007). Toxicity of the booster biocide Sea-Nine to the early developmental stages of the sea urchin *Paracentrotus lividus*. *Aquatic Toxicology*, 83(1), 52–61. doi:10.1016/j.aquatox.2007.03.011.

Bers, A., D'Souza, F., Klijnstra, J., Willemsen, P., and Wahl, M. (2006). Chemical defence in mussels: antifouling effect of crude extracts of the periostracum of the blue mussel *Mytilus edulis*. *Biofouling*, 22(4), 251–259. doi: 10.1080/08927010600901112.

Bhatt, I., and Tripathi, B. (2011). Interaction of engineered nanoparticles with various components of the environment and possible strategies for their risk assessment. *Chemosphere*, 82(3), 308–317. doi:10.1016/j.chemosphere.2010.10.011.

Bielmyer, G., Brix, K., and Grosell, M. (2008). Is Cl^- protection against silver toxicity due to chemical speciation? *Aquatic Toxicology*, 87(2), 81–87. doi:10.1016/j.aquatox.2008.01.004.

Biswas, P., and Wu, C. (2005). Nanoparticles and the Environment. *Air & Waste Management Association*, 55, 708–746. doi: 10.1080/10473289.2005.10464656.

Blasco, J., Chapman, P., Campana, O., and Hampel, M. (2016). Marine Ecotoxicology – Current Knowledge and Future Issues. Amsterdam: Elsevier/AP. ISBN: 9780128033722.

Boeck, G., Grosell, M., and Wood, C. (2001). Sensitivity of the spiny dogfish (*Squalus acanthias*) to waterborne silver exposure. *Aquatic Toxicology*, 54(3–4), 261–275. doi:10.1016/s0166-445x(00)00180-6.

Borodina, T., Grigoriev, D., Carillo, M., Hartmann, J., Moehwald, H., and Shchukin, D. (2014). Preparation of Multifunctional Polysaccharide Microcontainers for Lipophilic Bioactive Agents. *ACS Applied Materials & Interfaces*, 6, 6570−6578. doi: 10.1021/am406039r.

Braithwaite, R., and Fletcher, R. (2005). The toxicity of Irgarol 1051 and Sea-Nine 211 to the non-target macroalga *Fucus serratus* Linnaeus, with the aid of an image capture and analysis system.

Journal of Experimental Marine Biology and Ecology, 322(2), 111−121. doi:10.1016/j.jembe.2005.01.015.

Branscomb, E., and Rittschof, D. (1984). An investigation of low frequency sound waves as a means of inhibiting barnacle settlement. *Journal of Experimental Marine Biology and Ecology*, 79(2), 149–154. doi:10.1016/0022-0981(84)90215-6.

Burchardt, A., Carvalho, R., Valente, A., Nativo, P., Gilliland, D., Garcìa, C., Passarella, R., Pedroni, V., Rossi, F., and Lettieri, T. (2012). Effects of Silver Nanoparticles in Diatom *Thalassiosira pseudonana* and Cyanobacterium *Synechococcus* sp. *Environmental Science & Technology*, 46(20), 11336−11344. doi:10.1021/es300989e.

Burgess, J., Boyd, K., Armstrong, E., Jiang, Z., Yan, L., Berggren, M., May, U., Pisacane, T., Granmo, A. and Adams, D. (2003). The Development of a Marine Natural Product-based Antifouling Paint. *Biofouling*, 19(Supplement), 197–205. doi:10.1080/0892701031000061778.

Buzea, C., Pacheco, I., and Robbie, K. (2007). Nanomaterials and nanoparticles: Sources and toxicity. *Biointerphases*, 2(4), MR17–MR71. doi:10.1116/1.2815690.

Calabrese, A., and Nelson, D. (1974). Inhibition of embryonic development of the hard clam. *Mercenaria mercenaria*, by heavy metals. *Bulletin of environmental contamination and toxicology*, 11(1), 92–97.

Calabrese, A., Macinnes, J.., Nelson, D., and Miller, J. (1977). Survival and growth of bivalve larvae under heavy-metal stress. *Marine Biology*, 41(2), 179–184. doi:10.1007/bf00394024.

Candries, M. (2000). Paint systems for the marine industry. Notes to Complement the External Seminar on Antifoulings, Department of Marine Technology, University of Newcastle-upon-Tyne.

Canterford, G., and Canterford, D. (1980). Toxicity of Heavy Metals to the Marine Diatom *Ditylum Brightwellii* (West) Grunow: Correlation between Toxicity and Metal Speciation. *Journal of the Marine Biological Association of the United Kingdom*, 60(01), 227–242. doi:10.1017/s0025315400024280.

Cao, S., Wang, J., Chen, H., and Chen, D. (2010). Progress of marine biofouling and antifouling technologies. *Chinese Science Bulletin*, 56(7), 598–612. doi:10.1007/s11434-010-4158-4.

Cardin, J. (1981) Results of Acute Toxicity Tests Conducted with Silver at ERL, U.S.EPA, Narragansett.

Clement, J., and Jarrett, P. (1994). Antibacterial silver. *Metal-based drugs*, 1(5-6), 467–482. doi:10.1155/mbd.1994.467.

Costanza, R., dArge, R., deGroot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R., Paruelo, J., Raskin, R., Sutton, P., and Belt, M. (1997). The value of the world's ecosystem services and natural capital. *Nature*, 387, 253–260. doi:10.1038/387253a0.

Dafforn, K., Lewis, J., and Johnston, E. (2011). Antifouling strategies: History and regulation, ecological impacts and mitigation. *Marine Pollution Bulletin*, 62(3), 453–465. doi:10.1016/j.marpolbul.2011.01.012.

David, M. (2013). Measuring Engineered Nanomaterials in the Environment: A Consortium View of How to Address the Problem. *Environmental Engineering Science*, 30(3), 97–100. doi:10.1089/ees.2012.0327.

DCOIT Assessment Report (2014) Evaluation of active substances under the Regulation (EU) n°528/2012 concerning the making available on the market and use of biocidal products. Norway.

Demirel, Y., Turan, O., and Incecik, A. (2017). Predicting the effect of biofouling on ship resistance using CFD. *Applied Ocean Research*, 62, 100–118. doi: 10.1016/j.apor.2016.12.003.

Devilla, R., Brown, M., Donkin, M., Tarran, G., Aiken, J., and Readman, J. (2005). Impact of antifouling booster biocides on single microalgal species and on a natural marine phytoplankton community. *Marine Ecology Progress Series*, 286, 1–12. doi:10.3354/meps286001.

Dinnel, P., Stober, Q., Crumley, S., and Nakatani, R. (1982). Development of a Sperm Cell Toxicity Test for Marine Waters. *Aquatic Toxicology and Hazard Assessment*. doi:10.1520/stp36711s

Dinnel, P., Stober, Q., Link, J., Letourneau, M., Roberts, W., Felton, S., and Nakatani, R. (1983) Methodology and validation of a sperm cell toxicity test for testing toxic substances in marine waters. Final report Grant R/Tox-1, University of Washington, Seattle.

Dinnel, P., Link, J., Stober, Q., Letourneau, M., and Roberts, W. (1989). Comparative sensitivity of sea urchin sperm bioassays to metals and pesticides. *Archives of Environmental Contamination and Toxicology*, 18(5), 748–755. doi:10.1007/bf01225012.

Dorfman, D. (1977). Tolerance of *Fundulus heteroclitus* to different metals in salt waters. *Bulletin New Jersey Academy of Science*, 22, 21–23.

EC (European Comission) (2011). Commission recommendation of 18 October 2011 on the definition of nanomaterial. Official Journal of the European Union.

EPA (1992) Pesticide Ecotoxicity Database (Formerly: Environmental Effects Database (EEDB)) Environmental Fate and Effects Division, Washington, D.C..

EU (European Union) (2012). Regulation (EU) No 528/2012 of the european parliament and of the council of 22 may 2012 concerning the making available on the market and use of biocidal products. Official Journal of the European Union.

Evans, S., Leksono, T., and Mckinnell, P. (1995). Tributyltin pollution: A diminishing problem following legislation limiting the use of TBT-based anti-fouling paints. *Marine Pollution Bulletin*, 30(1), 14–21. doi:10.1016/0025-326x(94)00181-8.

Evans, S. (1999). TBT or not TBT? That is the question. *Biofouling*, 14(2), 117–129. doi:10.1080/08927019909378403.

Fewtrell, L. (2014). Silver: water disinfection and toxicity. Aberystwyth University, Aberystwyth.

Finnegan, M., Pittman, S., and Delorenzo, M. (2008). Lethal and Sublethal Toxicity of the Antifoulant Compound Irgarol 1051 to the Mud Snail *Ilyanassa obsoleta*. *Archives of Environmental Contamination and Toxicology*, 56(1), 85–95. doi:10.1007/s00244-008-9166-x.

Forbes, V. and Callow, P. (2002). Species Sensitivity Distributions Revisited: A Critical Appraisal. *Human and Ecological Risk Assessment: An International Journal*, 8(3), 473–492. doi: 10.1080/10807030290879781.

Forbes, V., and Forbes, T. (1994). Ecotoxicology in Theory and Practice. London: Chapman and Hall. ISBN: 0412435306.

Forget, K., Menasria, R., and Pavillon, J. (1995) Influence of the Organic Matter Content of Seawater on the Toxicity of Silver to Harpacticoid Copepod: *Tigriopus brevicornis*. *Journal de recherche oceanographique*, 20 (1–2), 63–69.

Fusetani, N., and Clare, A. (2006). Antifouling compounds. Berlin: Springer. ISBN: 978-3-540-30016-8.

Galante-Oliveira, S., Oliveira, I., Santos, J., Pereira, M., Pacheco, M., and Barroso, C. (2010). Factors affecting RPSI in imposex monitoring studies using *Nucella lapillus* (L.) as bioindicator. *Journal Of Environmental Monitoring*. 12, 1055–1063.

Gama, B., Pereira, R., and Coutinho, R. (2009). Bioincrustação marinha *in* Pereira, R., Soares-Gomes, A. (2009). Biologia Marinha (2º ed) (pp. 299–318). Rio de Janeiro: Interciência. ISBN: 9788571932135.

Garner, K., Suh, S., Lenihan, H., and Keller, A. (2015). Species Sensitivity Distributions for Engineered Nanomaterials. *Environmental Science & Technology*, 49(9), 5753–5759. doi:10.1021/acs.est.5b00081.

Gatidou, G., and Thomaidis, N. (2007). Evaluation of single and joint toxic effects of two antifouling biocides, their main metabolites and copper using phytoplankton bioassays. *Aquatic Toxicology*, 85(3), 184–191. doi:10.1016/j.aquatox.2007.09.002.

Georgantzopoulou, A., Balachandran, Y., Rosenkranz, P., Dusinska, M., Lankoff, A., Wojewodzka, M., Kruszewski, M., Cuignard, C., Audinot, J., Girija, S., Hoffman, L., and Gutleb, A. (2012). Ag nanoparticles: size- and surface-dependent effects on model aquatic organisms and uptake evaluation with NanoSIMS. *Nanotoxicology*, 7(7), 1168–1178. doi:10.3109/17435390.2012.715312.

Gottschalk, F., Sun, T., and Nowack, B. (2013). Environmental concentrations of engineered nanomaterials: Review of modeling and analytical studies. *Environmental Pollution*, 181, 287–300. doi:10.1016/j.envpol.2013.06.003

Gutner-Hoch, E., Martins, R., Oliveira, T., Maia, F., Soares, A., Loureiro, S., Piller, C., Preiss, I., Weis, M., Larroze, S., Teixeira, T., Tedim, J., Benayahu, Y. (Submitted). Antimacrofouling efficacy of innovative inorganic nanomaterials loaded with booster biocides. Journal of Marine Science and Engineering.

Handy, R., Owen, R., and Valsami-Jones, E. (2008). The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs. *Ecotoxicology*, 17(5), 315–325. doi:10.1007/s10646-008-0206-0.

Hansen, S. (2009). Regulation and Risk Assessment of Nanomaterials – Too Little, Too Late? PhD (Doctor of Philosophy) thesis, Department of Environmental Engineering, Technical University of Denmark, Denmark.

Harley, C., Randall-Hughes, A., Hultgren, K., Miner, B., Sorte, C., Thornber, C., Rodriguez, L., Tomanek, L., and Williams, S. (2006). The impacts of climate change in coastal marine systems. *Ecology Letters*, 9(2), 228–241. doi:10.1111/j.1461-0248.2005.00871.x.

Hassan, S. (2002). Protecting the marine environment from land-based sources of marine pollution: towards an effective International arrangement. PhD (Doctor of Philosophy) thesis, Faculty of Law, University of Wollongong.

Heitmuller, T. (1977) Acute toxicity of C-9211 to brown shrimp (*Penaeus aztecus*). Toxicity Test Report. EGandG, Bionomics, Marine Research Laboratory, Florida.

Hellio, C., and Yebra, D. (2009). Advances in Marine Antifouling Coatings and Technologies. Boca Raton: CRC Press. ISBN: 978-1-84569-631-3.

Hjorth, M., Haller, R., and Dahllöf, I. (2006). The use of 14C tracer technique to assess the functional response of zooplankton community grazing to toxic impact. *Marine Environmental Research*, 61(3), 339–351. doi:10.1016/j.marenvres.2005.11.003.

Henwood, C. (1888). Corrosion and Fouling of the Bottoms of Iron and Steel Ships, and Means of Preventing the Same. *Royal United Services Institution Journal*, 31(142), 919–936. doi:10.1080/03071848809416443.

Higuera-Ruiz, R., and Elorza, J. (2011). Shell thickening and chambering in the oysterCrassostrea gigas: natural and anthropogenic influence of tributyltin contamination. *Environmental Technology*, 32(6), 583–591. doi:10.1080/09593330.2010.506201.

Hook, S., and Fisher, N. (2001). Sublethal effects of silver in zooplankton: Importance of exposure pathways and implications for toxicity testing. *Environmental Toxicology and Chemistry*, 20(3), 568–574. doi:10.1002/etc.5620200316.

IMO (International Maritime Organization) (2001). International Convention on the Control of Harmful Anti-fouling Systems on Ships.

Jacobson, A., Mazza, L., Lawrence, L., Lawrence, B., Jackson, S., and Kesterson, A. (1993). Fate of an Antifoulant in an Aquatic Environment. *ACS Symposium Series Pesticides in Urban Environments*, 127–138. doi:10.1021/bk-1993-0522.ch012.

Jacobson, A., and Willingham, G. (2000). Sea-nine antifoulant: an environmentally acceptable alternative to organotin antifoulants. *Science of The Total Environment*, 258(1-2), 103–110. doi:10.1016/s0048-9697(00)00511-8.

Johnston, E., Mayer-Pinto, M., and Crowe, T. (2015). Review: Chemical contaminant effects on marine ecosystem functioning. *Journal of Applied Ecology*, 52(1), 140–149. doi:10.1111/1365-2664.12355.

Kahru, A., and Dubourguier, H. (2010). From ecotoxicology to nanoecotoxicology. *Toxicology*, 269 (2-3), 105–119. doi:10.1016/j.tox.2009.08.016.

Kawashima, Y. (1997) Acute Toxicity Test of RH-287 with Red Sea Bream. Test Report. Kurume Research Laboratories, Chemical Biotesting Center, Chemicals Inspection and Testing Institute, Japan.

Key, P., Chung, K., Hoguet, J., Sapozhnikova, Y., and Fulton, M. (2008). Effects of the anti-fouling herbicide Irgarol 1051 on two life stages of the grass shrimp, *Palaemonetes pugio*. *Journal of Environmental Science and Health*, Part B, 43(1), 50–55. doi:10.1080/03601230701734865.

Khodaparast, Z. (2015) Toxicity of silver nanoparticles and silver nitrate on *Nassarius reticulatus* larvae. MSc (Master of Science) thesis, University of Aveiro, Portugal.

Kooijman, S. (1987). A safety factor for LC50 values allowing for differences in sensitivity among species. *Water Research*, 21(3), 269–276. doi:10.1016/0043-1354(87)90205-3.

Koutsaftis, A., and Aoyama, I. (2007). Toxicity of four antifouling biocides and their mixtures on the brine shrimp Artemia salina. *Science of the Total Environment*, 387(1), 166–174. doi: 10.1016/j.scitotenv.2007.07.023.

Kristensen, J., Meyer, R., Laursen, B., Shipovskov, S., Besenbacher, F., and Poulsen, C. (2008). Antifouling enzymes and the biochemistry of marine settlement. *Biotechnology Advances*, 26(5), 471–481. doi:10.1016/j.biotechadv.2008.05.005.

Kumar, N., and Kumbhat, S. (2016). Essentials in Nanoscience and Nanotechnology. Hoboken: John Wiley & Sons. ISBN: 9781119096115.

Lejars, M., Margaillan, A., and Bressy, C. (2012). Fouling Release Coatings: A Nontoxic Alternative to Biocidal Antifouling Coatings. *Chemical Reviews*, 112(8), 4347–4390. doi:10.1021/cr200350v.

Lelieveld, H., Holah, J., and Gabric, D. (2016). Handbook of hygiene control in the food industry. Duxford: Woodhead Publishing. ISBN: 978-0-08-100197-4.

Lewin, R. (1984). Microbial adhesion is a sticky problem. *Science*, 224(4647), 375–377. doi:10.1126/science.6143401.

Loeb, G., and Neihof, R. (1975). Marine Conditioning Films. *Applied Chemistry at Protein Interfaces Advances in Chemistry*, 319–335. doi:10.1021/ba-1975-0145.ch016.

Lussier, S., and Cardin, J. (1985) Results of Acute Toxicity Tests Conducted with Silver at ERL, U.S.EPA, Narragansett.

Macken, A., Byrne, H., and Thomas, K. (2012). Effects of salinity on the toxicity of ionic silver and Ag-PVP nanoparticles to *Tisbe battagliai* and *Ceramium tenuicorne*. *Ecotoxicology and Environmental Safety*, 86, 101–110. doi:10.1016/j.ecoenv.2012.08.025.

Magin, C., Cooper, S., and Brennan, A. (2010). Non-toxic antifouling strategies. *Materials Today*, 13(4), 36–44. doi:10.1016/s1369-7021(10)70058-4.

Maia, F., Tedim, J., Lisenkov, A., Salak, A., and Zheludkevich, M. (2012) Silica nanocontainers for active corrosion protection. *Nanoscale*, 4, 1287–1298. doi:10.1016/j.electacta.2012.04.095.

Maia, F. (2015). Micro/nanoreservoirs for controlled release of active species in smart functional coatings. PhD (Doctor of Philosophy) thesis, University of Aveiro, Portugal.

Maia, F., Silva, A., Fernandes, S., Cunha, A., Almeida, A., Tedim, T., Zheludkevich, M., and Ferreira, M. (2015) Incorporation of biocides in nanocapsules for protective coatings used in maritime applications. *Chemical Engineering Journal*, 270, 150–157. doi: 10.1016/j.cej.2015.01.076.

Maltby, L., Blake, N., Brock, T., and Brink, P. (2005). Insecticide Species Sensitivity Distributions: Importance of Test Species Selection And Relevance To Aquatic Ecosystems. *Environmental Toxicology and Chemistry*, 24(2), 379–388. doi:10.1897/04-025r.1.

Marambio-Jones, C., and Hoek, E. (2010) A review of the antibacterial effects of silver nanomaterials and potential implications for human health and the environment. *Journal of Nanoparticle Research*, 12, 1531–1551. doi:10.1007/s11051-010-9900-y.

Martin, M., Osborn, K., Billig, P., and Glickstein, N. (1981). Toxicities of ten metals to *Crassostrea gigas* and *Mytilus edulis* embryos and *Cancer magister* larvae. *Marine Pollution Bulletin*, 12(9), 305–308. doi:10.1016/0025-326x(81)90081-3.

Martins, R., Oliveira, T., Santos, C., Kuznetsova, A., Ferreira, V., Avelelas, F., Caetano, A., Tedim, J., Ferreira, M., Freitas, R., Soares, A., and Loureiro, S. (2017). Effects of a novel anticorrosion engineered nanomaterial on the bivalve *Ruditapes philippinarum*. *Environmental Science: Nano*, 4(5), 1064–1076. doi: 10.1039/C6EN00630B.

Maruya, K., Dodder, N., Tang, C., Lao, W., and Tsukada, D. (2014). Which coastal and marine environmental contaminants are truly emerging?. *Environmental Science and Pollution Research*, 22(3), 1644–1652. doi:10.1007/s11356-014-2856-1.

Maurer-Jones, M., Gunsolus, I., Murphy, C., and Haynes, C., (2013). Toxicity of Engineered Nanoparticles in the Environment. *Analytical Chemistry*, 85(6), 3036–3049. doi:10.1021/ac303636s.

McFeters, G., Bond, P., Olson, S., and Tchan, Y. (1983). A comparison of microbial bioassays for the detection of aquatic toxicants. *Water Research*, 17(12), 1757–1762. doi:10.1016/0043-1354(83)90197-5.

McIntyre, R. (2012). Common nano-materials and their use in real world applications. *Science Progress*, 95(1), 1–22. doi:10.3184/003685012x13294715456431.

Menasria, R., and Pavillon, J. (1994). Toxic effects of two trace metals, copper and silver, on a crustacean harpacticoid copepod *Tigriopus brevicornis*(Mueller). Lethal and sublethal effects at different development stages. *Journal de recherche oceanographique*, 19(3), 157–165.

Mochida, K., Ito, K., Harino, H., Kakuno, A., and Fujii, K. (2006). Acute Toxicity of Pyrithione Antifouling Biocides and Joint Toxicity With Copper To Red Sea Bream (*Pagrus Major*) And Toy Shrimp (*Heptacarpus Futilirostris*). *Environmental Toxicology and Chemistry*, 25(11), 3058–3064. doi:10.1897/05-688r.1.

Mol, V., Raveendran, T., and Parameswaran, P. (2009). Antifouling activity exhibited by secondary metabolites of the marine sponge, Haliclona exigua (Kirkpatrick). *International Biodeterioration & Biodegradation*, 63(1), 67–72. doi:10.1016/j.ibiod.2008.07.001.

Mouneyrac, C., Mastain, O., Amiard, J., Amiard-Triquet, C., Beaunier, P., Jeantet, A., Smith, B., and Rainbow, P. (2003) Trace-metal detoxification and tolerance of the estuarine worm *Hediste*

diversicolor chronically exposed in their environment. *Marine Biology*, 143, 731–744. doi: 10.1007/s00227-003-1124-6.

Myers, J., Gunthorpe, L., Allinson, G., and Duda, S. (2006). Effects of antifouling biocides to the germination and growth of the marine macroalga, Hormosira banksii (Turner) Desicaine. *Marine Pollution Bulletin*, 52(9), 1048–1055. doi:10.1016/j.marpolbul.2006.01.010.

Nacci, D., Jackim, E., and Walsh, R. (1986). Comparative evaluation of three rapid marine toxicity tests: Sea urchin early embryo growth test, sea urchin sperm cell toxicity test and microtox. *Environmental Toxicology and Chemistry*, 5(6), 521–525. doi:10.1002/etc.5620050603.

Negri, A., Flores, F., Röthig, T., and Uthicke, S. (2011). Herbicides increase the vulnerability of corals to rising sea surface temperature. *Limnology and Oceanography*, 56(2), 471–485. doi:10.4319/lo.2011.56.2.0471.

Nelson, D., Calabrese, A., Nelson, B., Maclnnes, J., and Wenzloff, D. (1976). Biological effects of heavy metals on Juvenile Bay scallops, *Argopecten irradians*, in short-term exposures. *Bulletin of Environmental Contamination and Toxicology*, 16(3), 275–282. doi:10.1007/bf01685889.

Nendza, M. (2007). Hazard assessment of silicone oils (polydimethylsiloxanes, PDMS) used in antifouling-/foul-release-products in the marine environment. *Marine Pollution Bulletin*, 54(8), 1190–1196. doi:10.1016/j.marpolbul.2007.04.009.

Nguyen, T., Roddick, F., and Fan, L. (2012). Biofouling of water treatment membranes: a review of the underlying causes, monitoring techniques and control measures. *Membranes*, 2(4), 804–840. doi:10.3390/membranes2040804.

Okamura, H., Aoyama, I., Takami, T., Maruyama, T., Suzuki, Y., Matsumoto, M., Katsuyama, I., Hamada, J., Beppu, T., Tanaka, O., Maguire, R., Liu, D., Lau, L., and Pacepavicius, G. (2000). Phytotoxicity of the New Antifouling Compound Irgarol 1051 and a Major Degradation Product. *Marine Pollution Bulletin*, 40(9), 754–763. doi:10.1016/s0025-326x(00)00020-5.

Olsen, S., Pedersen, L., Laursen, M., Kiil, S., and Dam-Johansen, K. (2007). Enzyme-based antifouling coatings: a review. *Biofouling*, 23(5), 369–383. doi:10.1080/08927010701566384.

Panagoula, B., Panayiota, M., and Iliopoulou-Georgudaki, J. (2002). Acute Toxicity of TBT and Irgarol in *Artemia salina*. *International Journal of Toxicology*, 21(3), 231–233. doi:10.1080/10915810290096360.

Pedroso, M., Bersano, J., and Bianchini, A. (2007). Acute Silver Toxicity in The Euryhaline Copepod *Acartia Tonsa*: Influence of Salinity And Food. *Environmental Toxicology and Chemistry*, 26(10), 2158–2165. doi:10.1897/06-485r.1.

Pesch, C., and Hoffman, G. (1983) Interlaboratory Comparison of a 28-Day Toxicity Test with the Polychaete *Neanthes arenaceodentata*. *Aquatic Toxicology and Hazard Assessment*: Sixth Symposium. doi:10.1520/stp33525s.

Posthuma, L., Suter II, G., and Traas, T. (2002). Species Sensitivity Distributions in Ecotoxicology. Boca Raton: CRC Press. ISBN: 9781566705783.

Ray, P., Yu, H., and Fu, P. (2009). Toxicity and Environmental Risks of Nanomaterials: Challenges and Future Needs. *Journal of Environmental Science and Health*, 27(1), 1–35. doi:10.1080/10590500802708267.

Readman, J. (2005). Development, Occurrence and Regulation of Antifouling Paint Biocides: Historical Review and Future Trends. *The Handbook of Environmental Chemistry Antifouling Paint Biocides*, 5, 1–15. doi:10.1007/698_5_047.

Rosen, G., Osorio-Robayo, A., Rivera-Duarte, I., and Lapota, D. (2007). Comparison of Bioluminescent Dinoflagellate (QwikLite) and Bacterial (Microtox) Rapid Bioassays for the Detection of Metal and Ammonia Toxicity. *Archives of Environmental Contamination and Toxicology*, 54(4), 606–611. doi:10.1007/s00244-007-9068-3.

Salta, M., Wharton, J., Stoodley, P., Dennington, S., Goodes, L., Werwinski, S., Mart, U., Wood, R., and Stokes, K. (2010). Designing biomimetic antifouling surfaces. *Philosophical Transactions of the Royal Society A*, 368(1929), 4729–4754. doi:10.1098/rsta.2010.0195.

Scarlett, A., Donkin, M., Fileman, T., and Donkin, P. (1997). Occurrence of the marine antifouling agent irgarol 1051 within the Plymouth Sound locality: Implications for the green macroalga *Enteromorpha intestinalis*. *Marine Pollution Bulletin*, 34(8), 645–651. doi:10.1016/s0025-326x(96)00187-7.

Schimmel, S. (1981). Results: Interlaboratory comparison-acute toxicity tests using estuarine animals. US Environmental Protection Agency, Office of Research & Development, Environmental Research Laboratory.

Seery, C., Gunthorpe, L., and Ralph, P. (2006). Herbicide impact on *Hormosira banksii* gametes measured by fluorescence and germination bioassays. *Environmental Pollution*, 140(1), 43–51. doi:10.1016/j.envpol.2005.07.001.

Shade, W., Hurt, S., Jacobson, A., and Reinert, K. (1993). Ecological Risk Assessment of a Novel Marine Antifoulant. *Environmental Toxicology and Risk Assessment*, 1216, 381–408. doi: 10.1520/STP13169S.

Shaw, J., Wood, C., Birge, W., and Hogstrand, C. (1998). Toxicity of Silver to the Marine Teleost (*Oligocottus Maculosus*): Effects of Salinity And Ammonia. *Environmental Toxicology and Chemistry*, 17(4), 594–600. doi:10.1897/1551-5028(1998)017<0594:tosttm>2.3.co;2

Silva, P., Silva, A., Mendo, S., and Loureiro, S. (2014). Toxicity of tributyltin (TBT) to terrestrial organisms and its species sensitivity distribution. *Science of The Total Environment*, 466–467, 1037–1046. doi:10.1016/j.scitotenv.2013.08.002.

Sokolov, S.., Tschulik, K., Batchelor-Mcauley, C., Jurkschat, K., and Compton, R. (2015). Reversible or Not? Distinguishing Agglomeration and Aggregation at the Nanoscale. *Analytical Chemistry*, 87(19), 10033–10039. doi:10.1021/acs.analchem.5b02639.

Stauber, J., Binet, M., Bao, V., Boge, J., Zhang, A., Leung, K., and Adams, M. (2008). Comparison of the Qwiklite™ algal bioluminescence test with marine algal growth rate inhibition bioassays. *Environmental Toxicology*, 23(5), 617–625. doi:10.1002/tox.20400.

Sun, T., Gottschalk, F., Hungerbühler, K., and Nowack, B. (2014). Comprehensive probabilistic modelling of environmental emissions of engineered nanomaterials. *Environmental Pollution*, 185, 69–76. doi:10.1016/j.envpol.2013.10.004.

Tedim, J., Poznyak, S., Kuznetsova, A., Raps, D., Hack, T., Zheludkevich, M., and Ferreira, M. (2010). Enhancement of Active Corrosion Protection via Combination of Inhibitor-Loaded Nanocontainers. *ACS Applied Materials & Interfaces*, 2(5), 1528–1535. doi: 10.1016/j.electacta.2012.04.095.

Thain, J. (1986). Toxicity of TBT to Bivalves: Effects on Reproduction, Growth and Survival. *Oceans'86*, 1306–1313. doi:10.1109/oceans.1986.1160370.

Thomas, K. (2001). The environmental fate and behaviour of antifouling paint booster biocides: A review. *Biofouling*, 17(1), 73–86. doi:10.1080/08927010109378466.

Thorlaksen, P., Yebra, D., and Català, P. (2010). Hydrogel-based third generation fouling release coatings. Royal Belgian Institute of Marine Engineers.

Tsunemasa, N., and Okamura, H. (2010). Effects of Organotin Alternative Antifoulants on Oyster Embryo. *Archives of Environmental Contamination and Toxicology*, 61(1), 128–134. doi:10.1007/s00244-010-9598-y.

Vajtai, R. (2013). Springer Handbook of Nanomaterials. Berlin: Springer. ISBN: 978-3-642-20595-8.

Vance, M., Kuiken, T., Vejerano, E., Mcginnis, S., Hochella, M., Rejeski, D., and Hull, M. (2015). Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. *Beilstein Journal of Nanotechnology*, 6, 1769–1780. doi:10.3762/bjnano.6.181.

Van Vlaardingen, P., and Verbruggen, E. (2007). Guidance for the derivation of environmental risk limits within the framework of'International and national environmental quality standards for substances in the Netherlands'(INS). Revision 2007.

Willemsen, P., Overbeke, K., and Suurmond, A. (1998) Repetitive testing of TBTO, sea-nine 211 and farnesol using *Balanus amphitrite* (Darwin) cypris larvae: variability in larval sensitivity. *Biofouling*, 12, 133–147. doi:10.1080/08927019809378350.

Wilson, W., and Freeburg, L. (1980) Toxicity of Metals to Marine Phytoplankton Cultures. Environmental Research Laboratory, Office of Research and Development, US Environmental Protection Agency.

Xiong, H., Qi, S., Xu, Y., Miao, L., and Qian, P. (2009). Antibiotic and antifouling compound production by the marine-derived fungus Cladosporium sp. F14. *Journal of Hydro-environment Research*, 2(4), 264–270. doi:10.1016/j.jher.2008.12.002.

Xu, X., Wang, X., Li, Y., Wang, Y., and Wang, Y. (2010). Acute toxicity and synergism of binary mixtures of antifouling biocides with heavy metals to embryos of sea urchin *Glyptocidaris crenularis*. *Human & Experimental Toxicology*, 30(8), 1009–1021. doi:10.1177/0960327110385958

Yamada, H. (2007) Toxicity and Preliminary Risk Assessment of Alternative Antifouling Biocides to Aquatic Organisms. *The Handbook of Environmental Chemistry Antifouling Paint Biocides*, 213–226. doi:10.1007/698_5_056.

Yang, C., Song, G., Zhu, Q., Liu, S., and Xia, C. (2016). The influence of bacterial quorum-sensing inhibitors against the formation of the diatom-biofilm. *Chemistry and Ecology*, 32(2), 169–181.

Yebra, D., Kiil, S., and Dam-Johansen, K. (2004). Antifouling technology – past, present and future steps towards eficiente and environmentally friendly antifouling coatings. *Progress in Organic Coatings*, 50(2), 75–104. doi:10.1016/j.porgcoat.2003.06.001.

Ward, T., Kramer, J., Boeri, R., and Gorsuch, J. (2006). Chronic Toxicity of Silver to the Sea Urchin (*Arbacia Punctulata*). *Environmental Toxicology and Chemistry*, 25(6), 1568–1573. doi:10.1897/05-299r.1.

Wendt (2013) On the efficacy and ecotoxicity of antifouling biocides. Lethal and sublethal effects on target and non-target organisms. PhD (Doctor of Philosophy) thesis, University of Gothenburg, Sweden.

Wendt, I., Arrhenius, Å, Backhaus, T., Hilvarsson, A., Holm, K., Langford, K., Tunovic, T., and Blanck, H. (2013). Effects of Five Antifouling Biocides on Settlement and Growth of Zoospores from the Marine Macroalga *Ulva lactuca* L. *Bulletin of Environmental Contamination and Toxicology*, 91(4), 426–432. doi:10.1007/s00128-013-1057-9.

Wendt, I., Backhaus, T., Blanck, H., and Arrhenius, Å. (2016). The toxicity of the three antifouling biocides DCOIT, TPBP and medetomidine to the marine pelagic copepod Acartia tonsa. *Ecotoxicology*, 25(5), 871–879. doi:10.1007/s10646-016-1644-8.

WHOI (Woods Hole Oceanographic Institution) (1952). Marine fouling and its prevention – prepared for Bureau of Ships, Navy Deptartment. doi:10.1575/1912/191.

Wheeler, J., Grist, E., Leung, K., Morritt, D., and Crane, M. (2002). Species sensitivity distributions: data and model choice. *Marine Pollution Bulletin*, 45(1–12), 192–202. doi:10.1016/s0025-326x(01)00327-7.

Wijnhoven, S., Peijnenburg, W., Herberts, C., Hagens, W., Oomen, A,, Heugens, E., Roszek, B., Bisschops, J., Gosens, I., van de Meent, D., Dekkers, S., De Jong, W., van Zijverden, M., Sips, A., and Geertsma, R. (2009) Nano-silver – a review of available data and knowledge gaps in

human and environmental risk assessment. *Nanotoxicology* 3(2), 109–138. doi:10.1080/17435390902725914.

Williams, T. (2007). The mechanism of action of isothiazolone biocides. *PowerPlant Chemistry*, 9(1), 14.

Willingham, G., and Jacobson, A. (1996). Designing an Environmentally Safe Marine Antifoulant. *ACS Symposium Series*, 640, 224–233. doi:10.1021/bk-1996-0640.ch011.

Zhang, M., Cabane, E., and Claverie, J. (2007). Transparent antifouling coatings via nanoencapsulation of a biocide. *Journal of Applied Polymer Science*, 105(6), 3826–3833. doi:10.1002/app.26659.

Zhang, A.., Leung, K., Kwok, K., Bao, V., and Lam, M. (2008). Toxicities of antifouling biocide Irgarol 1051 and its major degraded product to marine primary producers. *Marine Pollution Bulletin*, 57(6–12), 575–586. doi:10.1016/j.marpolbul.2008.01.021.

Zheludkevich, M., Tedim, J., and Ferreira, M. (2012). “Smart” coatings for active corrosion protection based on multi-functional micro and nanocontainers. *Electrochimica Acta*, 82, 314–323. doi:10.1016/j.electacta.2012.04.095.

Zhou, F. (2015). Antifouling Surfaces and Materials – From Land to Marine Environment. Berlin: Springer. ISBN: 978-3-662-45204-2.

Chapter II

Toxicity of innovative anti-fouling nano-based solutions in marine species

2. Toxicity of innovative anti-fouling nano-based solutions in marine species

Joana Figueiredo[1], Roberto Martins[2], Tânia Oliveira[3], Frederico Maia[3], João Tedim[4], Amadeu Soares[2] and Susana Loureiro[2]

[1] Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[2] Department of Biology and CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[3] Smallmatek - Small Materials and Technologies, Lda., Rua Canhas, 3810-075 Aveiro, Portugal

[4] Department of Materials and Ceramic Engineering, CICECO, University of Aveiro, 3810-193 Aveiro, Portugal

2.1. Abstract

Biofouling is one of the most challenging problems for maritime industry that has been minimized through the application of coatings containing biocides. Currently, an innovative eco-friendly approach, namely the encapsulation of biocides in engineered nanomaterials in order to control their leaching rate, is being testing to decrease the toxicity of biocides to non-target species. An example of a nanomaterial used for this purpose are silica mesoporous nanocapsules (SiNC). The present study aims to assess the toxicity of three innovative solutions using this nanomaterial (SiNC loaded with DCOIT (SiNC-DCOIT), SiNC coated with silver (SiNC-Ag) and SiNC loaded with DCOIT and coated with silver (SiNC-DCOIT-Ag)) and of its major counterparts (DCOIT and silver) in order to assess if the encapsulation reduces the toxicity of biocides to non-target species maintaining the anti-fouling efficacy against target species. To achive this goal, three target (*Vibrio fischeri*, *Phaeodactylum tricornutum* and *Mytilus galloprovincialis*) and eight non-target (*Isochrysis galbana*, *Nannochloropsis gaditana*, *Brachionus plicatilis*, *Cerastoderma edule*, *Hediste diversicolor*, *Artemia salina*, *Palaemon varians* and *Paracentrotus lividus*) marine species were exposed to the test compounds, following standard protocols, with adaptations for some species. Globally, DCOIT and silver were very toxic or even extremely toxic to target and non-target species and the encapsulation reduced their toxicity for non-target species. Encapsulated biocides also had good efficacy towards fouler species. Thus, the present study demonstrated that the encapsulation of DCOIT and silver into silica nanocapsules seems to be a promising efficient and environmentally-friendly anti-fouling solution.

Keywords: Biofouling; DCOIT; Silver; Engineered nanomaterial; Silica nanocapsules; Exposure assessment

2.2. Introduction

Nanomaterials have been regarded the key for a new technological revolution in the 21st century (Husain and Khan, 2016). The number of commercial and industrial products containing nanomaterials have increased exponentially over the last few years due to their intrinsic properties improvement for several purposes (McIntyre, 2012). Recently, nanotechnology based approaches have been used to develop new innovative functional coatings for the maritime industry. One of these innovative solutions is the encapsulation of the active ingredients in engineered nanomaterials (ENMs) in order to prevent their direct interaction with coating ingredients and control their leaching rate during the early lifetime of conventional paints (Tedim et al., 2010; Maia et al., 2012; Zheludkevich et al., 2012; Maia et al., 2015; Avelelas et al., 2017). This strategy brings environmental and economic benefits and has been already successfully applied in coatings with anti-corrosion purposes, in which ENMs were used to encapsulate corrosion inhibitors (Tedim et al., 2010; Maia et al., 2012).

Anti-fouling biocides are commonly used in protective coatings for submerged structures (Arai et al., 2009; Zhou, 2015). Biofouling corresponds to the undesirable accumulation of fouling organisms (such as bacteria, algae, barnacles and mussels) in submerged surfaces with extensive ecological, environmental and economic impacts worldwide. These include, (a) an increase in vessels' weight and hulls' roughness and consequent increase of frictional drag, fuel consumption and CO_2 emissions; (b) an increase of the mass of fixed installations, distorting their initial configuration; (c) interference on the normal buoyancy of floating devices; (d) reduction of the durability of submerged structures; and (e) contribution for the dispersion of invasive/alien species through ballast water or ships' hulls around the world (WHOI, 1952; Jacobson and Willingham, 2000; Yebra et al., 2004; Gama et al., 2009; Hellio and Yebra, 2009; Cao et al., 2010).

In order to minimize these problems, in the recent past, organotin compounds were the most effective anti-fouling agent, however they were completely banned in 2008 due to the toxic and biomagnification effects (IMO, 2001; Yebra et al., 2004; Readman, 2005; Hellio and Yebra, 2009). As a consequence, a new generation of biocides (e.g. Copper and Zinc Pyrithione, Diuron, Zineb, Ziran, DCOIT, etc.) were developed with lower toxicity and persistence in the environment than organotin compounds (Thomas, 2001; Dafford et al., 2011; Zhou, 2015). DCOIT, also known as Sea-Nine 211, is one of these new biocides and was considered a promising solution with a broad-spectrum action (Jacobson and Willingham, 2000) but was also considered to have low environmental risk as it is biologically degraded in seawater in less than 24 hours.

However, according to various studies, booster biocides are still of concern due to their observed effects on non-target species specially at lower levels of marine food chains (Yebra et al., 2004; Dafforn et al., 2011; Price and Readman, 2013). As an example, Irgarol 1051, a recent booster biocide, was already banned of the list of allowed active compounds in the EU. Also, the increasingly strict environmental legislation and the consequently demands from industry to increase safety, lifetime and efficacy of paint formulations has led to the need to develop new strategies to improve these typical coatings based on biocides and make them more environmentally friendly (Almeida et al., 2007). Following this demand new nanotechnological-based solutions have been developed in which the booster biocides (e.g. DCOIT, pyrithiones) were encapsulated in ENMs, namely in silica mesoporous nanocapsules (SiNC-DCOIT: Maia, 2015; SiNC-Zn pyrithione and SiNC-Cu pyrithione: Avelelas et al., 2017) or in layered double hydroxides (LDH-Zn pyrithione and LDH-Cu pyrithione: Avelelas et al., 2017). Moreover, it has already been shown that the encapsulation of biocides reduced their toxicity relative to their free form (for SiNC-CuPT, SiNC-Zn-PT, LDH-CuPT and LDH-ZnPT) and that, in case of LDH-ZnPT, the anti-fouling efficacy was higher towards the mussel *Mytilus edulis* (Avelelas et al., 2017). Therefore, this technique seems to be a promising eco-friendly solution without compromising the efficacy of the active ingredients.

New nanomaterials are therefore being produced and scaled up, which is the case of the SiNC-DCOIT-Ag by the Portuguese company Smallmatek, Lda.. For this, silver was used as a coating in the SiNC-DCOIT nanocontainers, considering its well-known bactericidal properties (Wijnhoven et al., 2009; Nguyen et al., 2012; Fewtrell, 2014). Therefore, this coating will act at the early and first critical stage of biofouling, the formation of microbial biofilms (Abarzua and Jakubowski, 1995; Hellio and Yebra, 2009; Maia, 2015).

Although the efficacy of SiNC-DCOIT has been demonstrated in the bacteria *Vibrio fischeri* (Maia et al., 2015), the effects of this new material SiNC-DCOIT-Ag on non-target organisms have not yet been analyzed, as well as its efficacy. Therefore, the present study aims to assess the toxicity of the new engineered nanomaterial (SiNC-DCOIT-Ag) in marine target and non-target species and to compare its toxicity with the nanomaterials containing only one biocide (SiNC-DCOIT and SiNC-Ag) and with its individualized counterparts (empty SiNC, DCOIT and Ag).

2.3. Materials and methods

In this study a set of eleven relevant marine species was chosen based on their ecological relevance, availability and easy of harvest and laboratorial maintenance, adequate size, relatively short life cycles and sensitivity to contaminants. These characteristics make them adequate species for ecotoxicological studies, ensuring reliability, feasibility and cost-effectiveness in ecotoxicity practices. The selected species were also divided in: fouler/target species and non-fouler/non-target species. The selected target species were: the bacteria *Vibrio fischeri,* the diatom *Phaeodactylum tricornutum* and the bivalve *Mytilus galloprovincialis*. The non-target species were: the microalgae *Isochrysis galbana* and *Nannochloropsis gaditana*, the rotifer *Brachionus plicatilis*, the bivalve *Cerastoderma edule*, the polychaete *Hediste diversicolor*, the crustaceans *Artemia salina* and *Palaemon varians* and the echinoderm *Paracentrotus lividus*.

These species were exposed to SiNC, DCOIT, Ag^{+} (as $AgNO_3$), SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag following standard tests (with some adaptions). A quaternary ammonium compound, CTAB, is also used in the production of the silica nanocapsules to obtain a porous structure and allow the release of DCOIT. The presence of this compound in the final nanomaterial of the nanocapsules SiNC was not detected by Smallmatek Lda., demonstrating the efficacy of the different washing steps during the SiNC production. However, the toxicity of this compound was evaluated to understand its effects on organisms and to be used as a positive control in case of the appearance of residues of this compound. These results will be presented as supplementary data.

For all the performed tests, cultures and acclimation processes, artificial saltwater (ASW) with salinity 35 was used and prepared with reverse osmosis water and the artificial salt Tropic Marin® Pro Reef.

2.3.1. Acclimation in laboratory or cultures

Target species

The freeze-dried bacteria *V. fischeri* (Gammaproteobacteria: Vibrionaceae) was prepared by rehydratation with 1 mL of reconstitution solution and stored in the Microtox® analyzer at 4°C, according to the manufacturer.

The laboratory culture of *P. tricornutum* was prepared by adding Optimedium to artificial saltwater (previously filtered at 0.45 µm and autoclaved 20 min at 120ºC), according to the proportions indicated by the manufacturer (5 mL per liter of water). They were then kept in 250 mL Erlenmeyers with approximately 150 mL of culture medium, to allow gas exchange. The brown marine diatom has a unique feature of being enclosed within a cell wall made of silica, called a frustule so the medium was additionally supplemented with 0.03 g/L of sodium metasilicate nonahydrate ($Na_2SiO_3.9H_2O$). They were maintained in laboratory under room temperature (19±1ºC) and photoperiod conditions (16:8h (light:dark)) and daily shaked. In order to create the growth curve of the culture, aliquots were removed and the cell density monitored spectrophotometrically at 700 and 780 nm and correlated with cell density calculated using an optical microscope and a Newbauer chamber. This process was done every day until the cell number was stabilized. The wavelength that offered the best growth curve (considering the best adjustment given by the R^2, in this case 700 nm) was then used and considered for the subsequent toxicity tests. The regression equation between cell density (y) and OD_{700} (x) was calculated as $y = 10^{-08}x + 0.036$ ($R^2 = 0.94$) and initial cell density was 10^6 cells/ml.

Juvenile *M. galloprovincialis* (Bivalvia: Mytilidae) mussels (2–3 cm length) were collected at Costa Nova, Ílhavo, Portugal (40°36'56.1"N, 8°45'14.5"W), in September 2016. The acclimation in laboratory conditions was carried out for 72 h, with continuous aeration, at a temperature of 19±1ºC, 16:8 h (light:dark) photoperiod and without food.

Non-target species

Microalgae cultures of *I. galbana* (Prymnesiophyceae: Isochrysidaceae) and *N. gaditana* (Eustigmatophyceae: Monodopsidaceae) were maintained in laboratory under similar conditions to the above described for the diatoms, but for these species the medium was not supplemented with sodium metasilicate nonahydrate. The methodology to create the growth curve of cultures was also similar. The regression equation between

cell density (y) and OD_{780} (x) was calculated as $y = 10^{-08}x + 0.046$ ($R^2 = 0.97$) for *I. galbana* and $y = 10^{-08}x + 0.055$ ($R^2 = 0.90$) for *N. gaditana*. Initial cell density was 10^5 cells/ml for both species.

Rotifers (*B. plicatilis* (Monogononta: Brachionidae)) were obtained through the RotoxKit M® and the cysts were hatched in a climatic chamber with a temperature of 25±1ºC, continuous light and using artificial saltwater with salinity 20, obtained by adding 4.3 mL of deionized water to 5.7 mL of artificial saltwater at 35.

Dry artemia (*A. salina* (Branchiopoda: Artemiidae)) cysts were hydrated during 30 min in 300 mL of reverse osmosis water with hard aeration. Afterward, a sample was observed in a binocular lens and, after checking their full hydratation, 700 mL of artificial saltwater were added to correct the salinity to 25. After 16-24 h, organisms hatched and were washed with new artificial saltwater before use. In these study, *A. salina* was used in the stage second-third instar, reached after > 24 h.

Adult cockles (*C. edule* (Bivalvia: Cardiidae)) (average total body mass=9±3.31 g) and polychaetes (*H. diversicolor* (Polychaeta: Nereididae)) (average body mass=0.27±0.08 g; average length=6±1 cm) were collected in the same local of Ria de Aveiro (40°38'15.1"N, 8°44'15.0"W) in October and December 2016, respectively. Juvenile shrimps (*P. varians* (Malacostraca: Palaemonidae)) (average body mass=205±45.6 mg) were collected in another location of Ria de Aveiro (40°38'33.9"N, 8°39'47.0"W) in October 2016. These species were acclimated to laboratory conditions in the same way as mussels (*M. galloprovincialis*) but *P. varians* were additionally fed *ad libitum* with TetraMin®.

Sea urchins (*P. lividus* (Echinoidea: Parechinidae)) were collected in Figueira da Foz in October 2016 (40°10'09"N, 8°53'25.0"W) and acclimated in the laboratory in the same conditions as other species collected in the field, but additionally fed *ad libitum* with *Ulva* sp.. Gametes were obtained by inducing spawning by injecting 0.5-1.5 mL of 0.5M KCl into the coelomic cavity of the organisms. After several minutes the spawning occurred and the gametes of at least two males and two females were mixed in artificial saltwater. Using an optical microscope, the egg fertilization was verified and the amount of eggs in solution was adjusted, in approximate ratio of 5 fertilized eggs per µL.

2.3.2. Ecotoxicity testing

All tests were carried out in artificial saltwater filtered at 0.45 µm, at the temperature of 19±1ºC and photoperiod of 16:8 h (light:dark), except for *Vibrio fischeri* – Microtox® test conditions; *Brachionus plicatilis* and *Artemia salina* – kept at temperature of 25±1ºC, at the dark. There was no replacement of medium during the exposure testing period. Exposure concentration ranges can be consulted in Table 2.1S. Fresh stock solutions were prepared with filtered (0.45 µm) artificial saltwater and dried powders before each exposure test. All tested compounds were dried at 140 ºC (NMs were provided as slurry, while DCOIT was in a xylene solution).

Whenever possible, preliminary range finding tests were carried out to determine the more accurate range of concentrations to be tested and obtain a more reliable L/E/IC_{50} value. In the case of the species *M. galloprovincialis*, *C. edule*, *P. varians* and *P. lividus* only one test was performed for each compound due to the low availability of organisms in the field, to the low variability between replicates, and to the effects being detected already at the lower levels of the range-finding tests. Due to this last reason, for some compounds the tests with the species *P. tricornutum* (SiNC, CTAB and SiNC-Ag), *N. gaditana* (CTAB) and *H. diversicolor* ($AgNO_3$) were not repeated.

Target species

Bacteria – *Vibrio fischeri*

This species is a bioluminescent bacteria, and the exposure to a toxic substance causes a disturbance on its respiratory process and results in reduction of light emission. Tests performed with this species provide biologically relevant information on the toxicity of chemical compounds (Férard and Blaise, 2013), that will serve as basis for the creation of the experimental design of the tests performed with more complex species. This is of special importance when there is no information on the effects of the chemical compound to be tested. The Microtox® test was performed according to the standard protocol recommended by the manufacturer that adheres to the standard guidelines ISO 11348-3 (2007) and ASTM D5660. A dilution series of the stock solutions was prepared in sodium chloride solution (2% NaCl). The inhibition of luminescence after 5 minutes of exposure times was determined and the median inhibitory concentration (IC_{50}) derived. Only for silver the IC_{50} was calculated after 15 minutes of exposure.

Diatoms – *Phaeodactylum tricornutum*

The toxicity testing for *P. tricornutum* followed the guideline OECD 201 (2011) and ISO 10253 (2016) with adaptations. For each compound a 24-well microplate was used and five concentrations plus one negative control, with four replicates per treatment were tested. Each replicate contained 20 µL of the contaminant and 1980 µL of microalgae sample. Each day of the test cell density was measured spectrophotometrically at 700 nm and, in order to have a homogenized measurement, each well was resuspended with the help of a micropipette, immediately before each reading. The growth inhibition at 72 hours was then calculated, according to the following equation (where *% I_r* – percent inhibition in average specific growth rate; *μC* – mean value for average specific growth rate (µ) in the control group; *μT* – average specific growth rate for the treatment replicate):

$$\% \, I_r = \frac{\mu_c - \mu_T}{\mu_c} \times 100$$

Bivalves – *Mytilus galloprovincialis*

For the acute toxicity testing with *M. galloprovincialis* the guideline ASTM E724-98 was followed with adaptations (Gutner-Hoch et al., submitted). The tests were performed in 6-well microplates and for each compound five concentrations plus one negative control, with twelve replicates per treatment were tested. Each replicate contained 10 mL of medium (with the respective concentration) and one mussel. Mortality was checked daily till a total of 72 hours.

Non-target species

Microalgae – *Isochrysis galbana* and *Nannochloropsis gaditana*

The species *Isochrysis galbana* (Prymnesiophyceae: Isochrysidaceae) and *Nannochloropsis gaditana* (Eustigmatophyceae: Monodopsidaceae) are unicellular golden-brown and green marine microalgae, respectively. In these cases, the methodology was similar to the above described for the diatoms. For these species, cell density was measured spectrophotometrically at 780 nm. The growth inhibition was calculated in the same way as stated for the diatom.

Rotifers – *Brachionus plicatilis*

For the acute toxicity testing with *B. plicatilis* the standard protocol Rotoxkit M (Microbiotests®), that adheres to the standard protocol ISO 19820 (2016), was followed. For each compound a 24-well microplate was used and five concentrations plus a negative control, with three replicates per treatment were used. Each replicate contained 300 µL of medium and five rotifers. After 24 hours rotifers mortality/immobilization was verified, being defined as the total absence of movement (i.e., swimming activity) after observation for approximately 10 seconds. Therefore, dead and immobile organisms were reported.

Bivalves – *Cerastoderma edule*

For the acute toxicity testing with *C. edule* the guideline ASTM E724-98 was followed with adaptations (Martins et al., 2017). Tests were performed in 200 mL glass flasks and for each compound five concentrations plus a negative control, with five replicates per treatment were tested. Each replicate contained 150 mL of medium and one animal. Mortality was checked daily till a total of 96 hours. pH, dissolved oxygen and temperature values were measured during the course of the test (pH = 7.83±0.14; OD = 81.2±1.0%; T = 19±1ºC).

Polychaetes – *Hediste diversicolor*

The acute toxicity testing for *H. diversicolor* followed the guidelines EPA 712–C–96–136 (1996) and ASTM E729 (2002), with adaptations. Tests were performed in 200 mL glass flasks and for each compound five concentrations plus a negative control, with five replicates per treatment were tested. Each replicate contained 150 mL of medium and one animal. Mortality was checked for 96 hours.

Crustacean – *Artemia salina*

In order to evaluate the acute toxicity of the test compounds in *A. salina* the standard protocol ASTM E1440-91 (2012) was adapted. For each compound a 24-well microplate was used and five concentrations plus a negative control, with three replicates per treatment were tested. Each replicate contained 1 mL of medium and ten animals. After 48 hours mortality/immobilization was assessed, which is defined as total absence of movement (i.e. swimming activity and movement of the appendices) after mechanical stimulation and observation for approximately 10 seconds.

Crustacean – *Paleamon varians*

In order to evaluate the acute toxicity of the compounds in *P. varians*, the guideline EPA 712–C–96–136 (1996) was adapted. The tests were performed in 200 mL glass flasks and for each compound four concentrations plus a negative control, with five replicates per treatment were tested. Each replicate contained 150 mL of medium and three animals. Mortality was checked daily for 96 hours. pH, dissolved oxygen and temperature values were measured during the course of the test (pH = 7.97±0.11; OD = 81.0±1.3%; T = 19±1ºC).

Echinoderm species – *Paracentrotus lividus*

In order to evaluate the toxicity of the compounds *P. lividus* the guideline EPS 1/RM/27 (1992). For each compound a 24-well microplate was used and five concentrations plus a negative control, with four replicates per treatment were tested. Each replicate contained 20 µL of fertilized eggs (~ 100 eggs) and 1980 µL of the contaminant. After 24 and 48 hours the larval development was verified with an optical microscope. Approximately 48 hours after fertilization, juveniles emerge from the egg as a small gelatinous larva, called pluteus, which at its maximum size reaches a few millimeters in length, with bilateral symmetry, from whose body emerge 4-6 appendices. The endpoint of this test was to determine the number of live pluteus larvae at 48 hours.

2.3.3. Statistical analysis

Data normality and homoscedasticy were tested with the Shapiro-Wilks and Bartlett's tests, respectively. If these assumptions were checked, a one-way analysis of variance (one-way ANOVA) test was performed with the software *SigmaPlot v12.5* to evaluate significant differences between treatments and control ($p<0.05$). If differences were present, the ANOVA was followed by the Dunnett test. If data failed the normality or the homoscedastic assumptions, data transformation (i.e., Square, Ln, Log_{10}, Reciprocal, Exponential and Square Root) was carried out. If these transformations did not fulfil the aim for normality and homoscedasticy, a one-way ANOVA on Ranks was performed followed by a Dunn's test. NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) values were derived from these tests.

The $L/E/IC_{50}$ (i.e., the concentration that caused 50% mortality, effects or inhibition) were determined by a non-linear regression with the software *Graphpad Prism* v.6.0. For each compound and species, the non-linear regression equation that best fits the data was chosen, considering the R^2 value, the absolute Sum of Squares and the 95% Confidence Intervals. Results were transposed to classify the toxicity of each compound according to the EC Directive 93/67/EEC scheme, adapted for ENMs, from extremely toxic to non-toxic (Blaise et al., 2008). Categories are divided as: Extremely toxic: <0.1 mg/L; Very toxic: 0.1 - 1 mg/L; Toxic: 1 – 10 mg/L; Harmful: 10– 100 mg/L; Non-toxic: >100 mg/L.

In order to fulfil one of the aims of the study, i.e. to compare the toxicity between the free and encapsulated active ingredient, a toxicity ratio (TR) was also calculated, indicating whether the encapsulated active compound is more (TR < 1) or less (TR > 1) toxic than the free compound, according to the following equation:

$$\text{Toxicity ratio (TR)} = \frac{\text{L/E/IC}_{50}\text{(encapsulated form)}}{\text{L/E/IC}_{50}\text{(free form)}}$$

2.4. Results

The figures 2.1 to 2.3 and 2.4 to 2.11 show the dose response curves of the target and non-target marine species, respectively, used in the present study, to the tested compounds SiNC, DCOIT, Ag, SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag. The tables 2.1 to 2.11 show the corresponding L/E/IC_{50}, LOEC and NOEC values.

All tested compounds caused mortality, effects or inhibition (depending on the species) in a concentration-dependent manner, i.e., the effects for each endpoint increases as the concentration of the compounds increases.

Globally, SiNC was the less toxic compound, being harmful or non-toxic for five tested species, while both biocides (in its free form) were very toxic or even extremely toxic towards target and non-target species (Table 2.12). Considering the novel ENMs as heterocompounds (SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag), they were generally toxic to the tested species. The diatom *P. tricornutum* and the mussel *M. galloprovincialis* were the most and less sensitive species, respectively, to SiNC-DCOIT and SiNC-DCOIT-Ag. Regarding SiNC-Ag, the echinoderm *P. lividus* and the crustacean *A. salina* were the most and less sensitive species, respectively. Although the overall toxicity category of this new nanomaterials is toxic, for the majority of the species the encapsulation of the active compounds (DCOIT and Ag) reduced their toxicity comparing to the free forms, as shown by the toxicity ratio (Table 2.13). Above mentioned results are based on the whole compound, therefore results will be also presented based on each active ingredient compound, by calculating the concentration of the active compound being used for discussion and comparison.

Target species

Bacteria – *Vibrio fischeri*

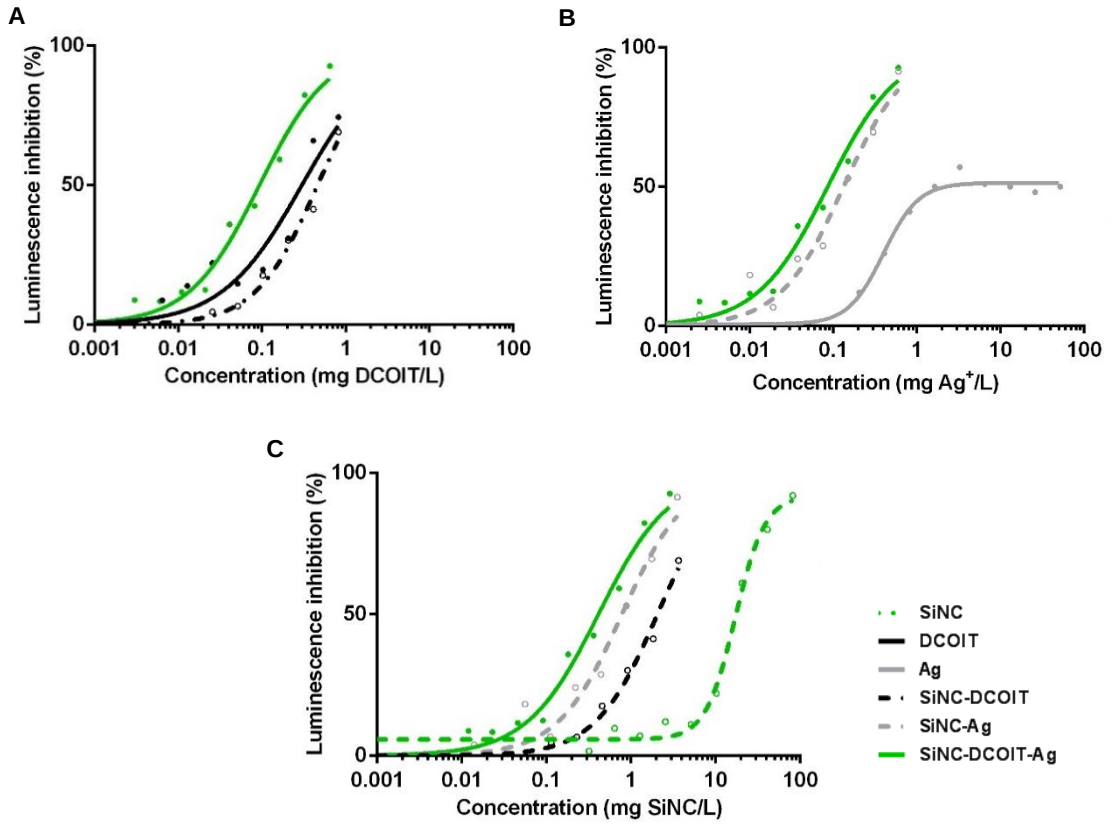


Figure 2.1 – Dose-response curves for *Vibrio fischeri* luminescensce inhibition exposed to: (A) – DCOIT, SiNC-DCOIT and SiNC-DCOIT-Ag (both expressed as DCOIT content); (B) Ag, SiNC-Ag and SiNC-DCOIT-Ag (both expressed as Ag content); (C) SiNC, SiNC-DCOIT, SiNC-Ag, SiNC-DCOIT-Ag (expressed as SiNC content). Data are presented as percentage mean values ± standard error.

Figure 2.1 and Table 2.1 show the results of the exposure of *V. fischeri* to the tested compounds. SiNC was the least toxic compound to this species (IC_{50} = 17.1 mg SiNC/L), being classified as harmful, and DCOIT was the one presenting higher toxicity (IC_{50} = 0.299 mg DCOIT/L), classified as extremely toxic (Table 2.12). The encapsulation of DCOIT in the silica nanocapsules (SiNC-DCOIT) was 1.5-fold less toxic than its free form (Table 2.13). Despite this decrease, DCOIT remains categorized as very toxic when encapsulated. SiNC-DCOIT-Ag demonstrated a powerful bactericidal activity against *V. fischeri* (the encapsulation increased the toxicity of both free biocides). Silver was also 3-fold more toxic in SiNC-Ag than the free form, being classified as very toxic in both forms. Silica nanocapsules were always more toxic loaded with the biocides comparing to their unloaded form.

Table 2.1 – No observed effect concentration (NOEC), lowest observed effect concentration (LOEC) and medium inhibition concentration values (IC_{50}) for the bacteria *Vibrio fischeri* during an exposure of 5 min to SiNC, DCOIT, Ag, SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag. Ag^+ – results for 15 minutes; n.d – not determined.

Contaminant	Units	IC_{50}	95% CI	NOEC	LOEC	Statistics
SiNC	mg SiNC/L	17.1	13.4 – 21.9	n.d	n.d	n.d
DCOIT	mg DCOIT/L	0.299	0.190 – 0.469	n.d	n.d	n.d
SiNC-DCOIT	mg DCOIT/L	0.459	0.377 – 0.559	n.d	n.d	n.d
	mg SiNC/L	2.05	1.68 – 2.50	n.d	n.d	n.d
	mg SiNC-DCOIT/L	2.51	2.06 – 3.06	n.d	n.d	n.d
Ag^+	mg Ag^+/L	0.397	0.295 – 0.534	n.d	n.d	n.d
SiNC-Ag	mg Ag^+/L	0.132	0.098 – 0.179	n.d	n.d	n.d
	mg SiNC/L	0.780	0.577 – 1.05	n.d	n.d	n.d
	mg SiNC-Ag/L	1.14	0.731 – 1.34	n.d	n.d	n.d
SiNC-DCOIT-Ag	mg SiNC/L	0.412	0.327 – 0.518	n.d	n.d	n.d
	mg DCOIT/L	0.093	0.075 – 0.116	n.d	n.d	n.d
	mg Ag^+/L	0.086	0.068 – 0.108	n.d	n.d	n.d
	mg SiNC-DCOIT-Ag/L	0.646	0.512 – 0.806	n.d	n.d	n.d

Diatom – *Phaeodactylum tricornutum*

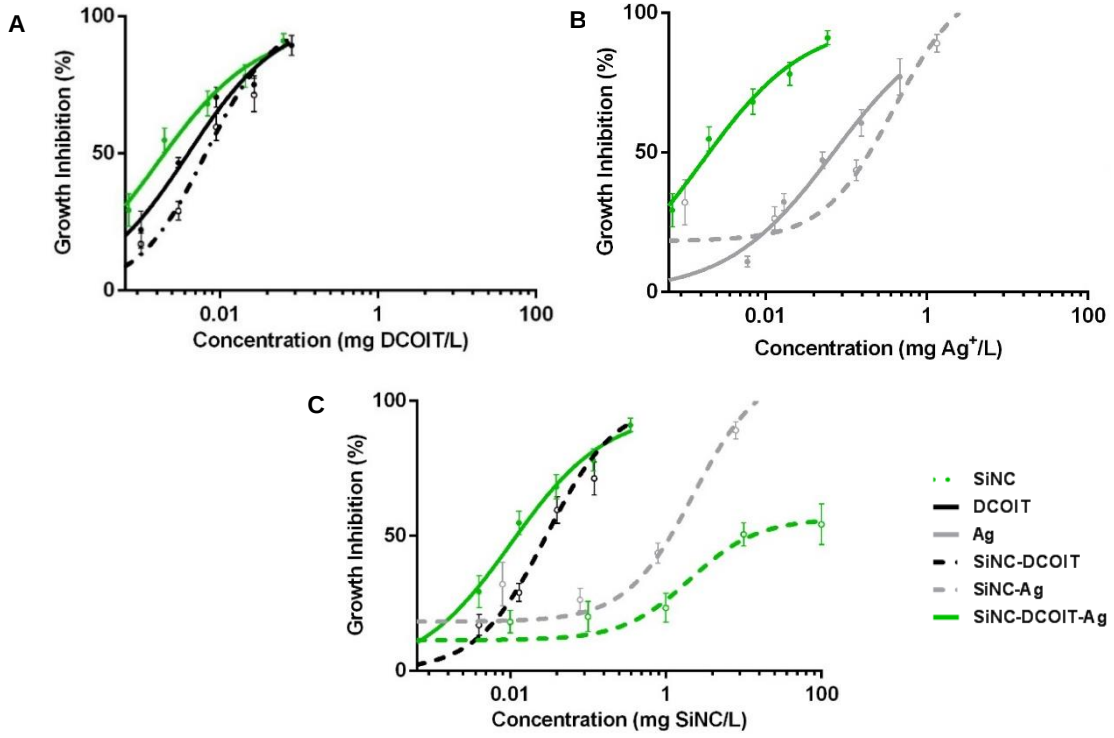


Figure 2.2 – Dose-response curves of *Phaeodactylum tricornutum* growth inhibition exposed to: (A) – DCOIT, SiNC-DCOIT and SiNC-DCOIT-Ag (both expressed as DCOIT content); (B) Ag, SiNC-Ag and SiNC-DCOIT-Ag (both expressed as Ag content); (C) SiNC, SiNC-DCOIT, SiNC-Ag, SiNC-DCOIT-Ag (expressed as SiNC content). Data are presented as percentage mean values ± standard error.

Figure 2.2 and Table 2.2 show the results of the exposure of *P. tricornutum* to the tested compounds. SiNC-Ag was the least toxic compound to this species (IC_{50} = 2.87 mg SiNC-Ag/L), but still classified as toxic, and DCOIT was the chemical that presented higher toxicity (IC_{50} = 0.004 mg DCOIT/L), classified as extremely toxic (Table 2.12). DCOIT remained extremely toxic in both encapsulated forms (SiNC-DCOIT and SiNC-DCOIT-Ag) (Table 2.13). However, in case of SiNC-DCOIT, the free biocide was almost 2-fold more toxic than the encapsulated form. Silver was also extremely toxic for the diatom as a coating of SiNC-DCOIT-Ag, being more toxic in this form comparing with the free form. In the nanomaterial SiNC-Ag, silver was 5.5-fold less toxic than the free biocide by itself, even though they are both in the category of very toxic. Silica nanocapsules were always more toxic when loaded with the active compounds, comparing to their unloaded form.

Table 2.2 – No observed effect concentration (NOEC), lowest observed effect concentration (LOEC) and medium inhitibition concentration values (IC_{50}) for the microalgae *Phaeodactylum tricornutum* during an exposure of 72 h to SiNC, DCOIT, Ag, SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag.

Contaminant	Units	IC_{50}	95% CI	NOEC	LOEC	Statistics
SiNC	mg SiNC/L	2.03	0.513 – 8.04	0.010	0.100	F=16.9; p=<0.001
DCOIT	mg DCOIT/L	0.004	0.003 – 0.005	0.003	0.009	H=21.6; p=<0.001
SiNC-DCOIT	mg DCOIT/L	0.007	0.005 – 0.009	0.001	0.003	F=42.7; p=<0.001
	mg SiNC/L	0.029	0.020 – 0.041	0.004	0.013	
	mg SiNC-DCOIT/L	0.036	0.025 – 0.051	0.005	0.016	
Ag^+	mg Ag^+/L	0.070	0.054 – 0.091	< 0.006	0.006	F=139.0; p=<0.001
SiNC-Ag	mg Ag^+/L	0.387	0.170 – 0.879	< 0.003	0.001	F=73.6; p=<0.001
	mg SiNC/L	2.26	0.996 – 5.13	< 0.008	0.008	
	mg SiNC-Ag/L	2.87	1.26 – 6.51	< 0.022	0.007	
SiNC-DCOIT-Ag	mg SiNC/L	0.010	0.005 – 0.021	< 0.004	0.004	F=69.5; p=<0.001
	mg DCOIT/L	0.002	0.001 – 0.003	< 0.001	0.0007	
	mg Ag^+/L	0.002	0.001 – 0.003	< 0.001	0.0007	
	mg SiNC-DCOIT-Ag/L	0.012	0.006 – 0.024	< 0.007	0.005	

Bivalve – *Mytilus galloprovincialis*

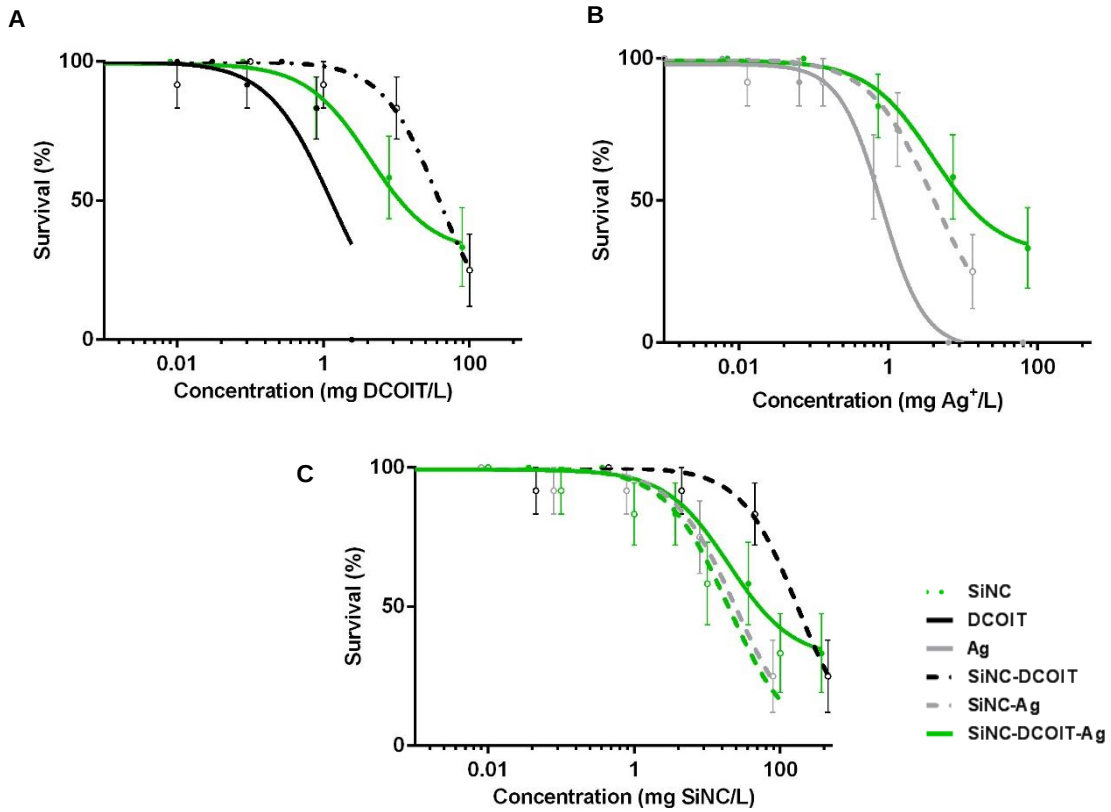


Figure 2.3 – Dose-response curves of *Mytilus galloprovincialis* survival exposed to: (A) – DCOIT, SiNC-DCOIT and SiNC-DCOIT-Ag (both expressed as DCOIT content); (B) Ag, SiNC-Ag and SiNC-DCOIT-Ag (both expressed as Ag content); (C) SiNC, SiNC-DCOIT, SiNC-Ag, SiNC-DCOIT-Ag (expressed as SiNC content). Data are presented as percentage mean values ± standard error.

Figure 2.3 and Table 2.3 show the results of the exposure of *M. galloprovincialis* to the tested compounds. SiNC-DCOIT was the least toxic compound to this species (LC_{50} = 210 mg SiNC-DCOIT/L), globally classified as non-toxic, and free Ag^+ was the chemical that presented higher toxicity, classified as very toxic (LC_{50} = 0.820 mg Ag^+/L) (Table 2.12). The encapsulation of both biocides in silica nanocapsules (SiNC-DCOIT, SiNC-Ag and SINC-DCOIT-Ag) reduced their toxicity, comparing with their free forms (Free DCOIT was 3-fold and 30-fold more toxic than SiNC-DCOIT-Ag and SiNC-DCOIT, respectively; Free Ag^+ was 5-fold less toxic comparing with the two encapsulation forms) (Table 2.13). Despite this reduction, the biocides in their encapsulated form remained in the extremely toxic category, such as in their free form, except in the case of SiNC-Ag (in which the corresponding biocide became very toxic when encapsulated). The toxicity of loaded silica nanocapsules were also reduced comparatively to the "empty" nanocapsule.

Table 2.3 – No observed effect concentration (NOEC), lowest observed effect concentration (LOEC) and medium lethal concentration values (LC_{50}) for the bivalve *Mytilus galloprovincialis* during an exposure of 72 h to SiNC, DCOIT, Ag, SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag.

Contaminant	Units	LC_{50}	95% CI	NOEC	LOEC	Statistics
SiNC	mg SiNC/L	19.5	9.09 – 41.8	10.0	100	H=24.4; p=<0.001
DCOIT	mg DCOIT/L	1.27	0.865 – 1.87	0.810	2.43	H=65.6; p=<0.001
SiNC-DCOIT	mg DCOIT/L	38.5	20.8 – 71.4	10.0	100.0	H=32.7; p=<0.001
	mg SiNC/L	172	92.6 – 319	44.6	446	
	mg SiNC-DCOIT/L	210	113 – 390	54.6	546	
Ag^+	mg Ag^+/L	0.820	0.476 – 1.41	0.635	6.35	H=55.4; p=<0.001
SiNC-Ag	mg Ag^+/L	4.11	2.109 – 8.02	1.34	13.4	H=31.1; p=<0.001
	mg SiNC/L	24.3	12.4 – 47.3	7.89	78.9	
	mg SiNC-Ag/L	30.7	15.8 – 59.9	10	100	
SiNC-DCOIT-Ag	mg SiNC/L	19.7	4.22 – 92.12	36.4	364	H=27.6; p=<0.001
	mg DCOIT/L	4.27	0.914 – 19.98	7.89	78.9	
	mg Ag^+/L	3.96	0.847 – 18.5	7.31	73.1	
	mg SiNC-DCOIT-Ag/L	29.7	6.35 – 139	54.8	548	

Non-target species

Microalgae – *Isochrysis galbana*

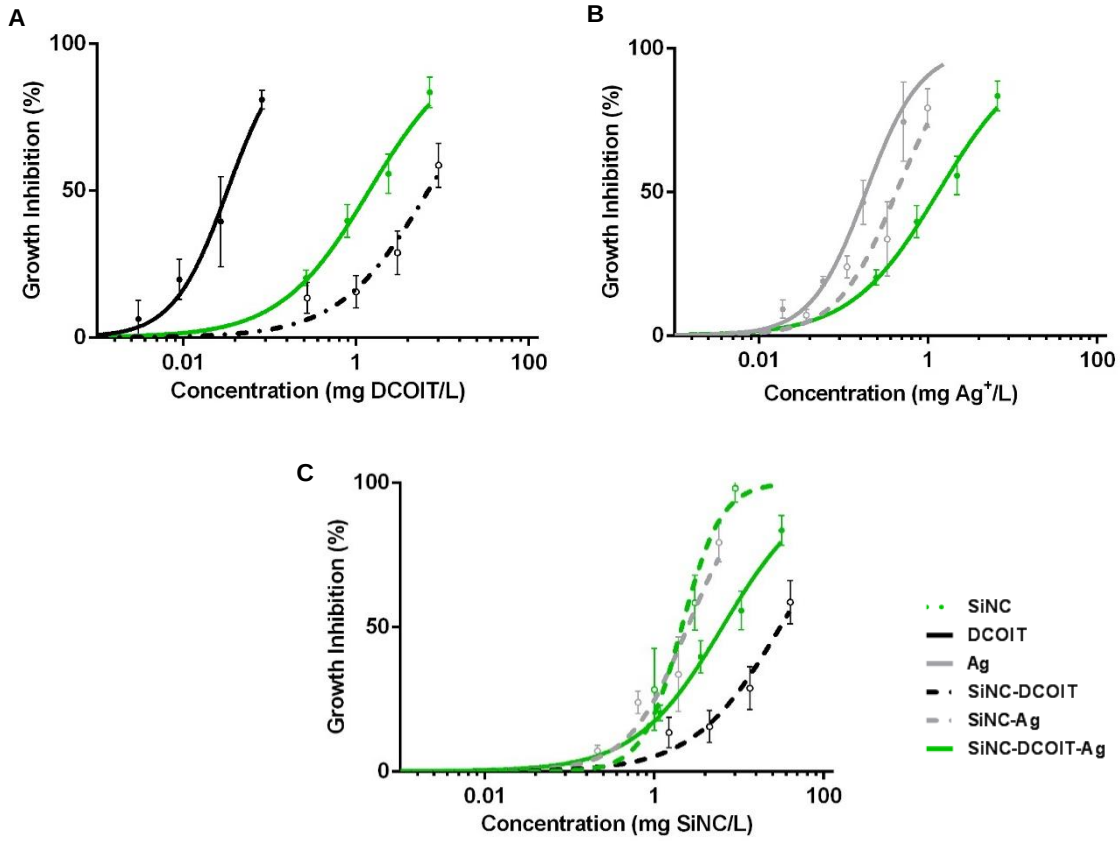


Figure 2.4 – Dose-response curves of *Isochrysis galbana* growth inhibiton exposed to: (A) – DCOIT, SiNC-DCOIT and SiNC-DCOIT-Ag (both expressed as DCOIT content); (B) Ag, SiNC-Ag and SiNC-DCOIT-Ag (both expressed as Ag content); (C) SiNC, SiNC-DCOIT, SiNC-Ag, SiNC-DCOIT-Ag (expressed as SiNC content). Data are presented as percentage mean values ± standard error.

Figure 2.4 and Table 2.4 show the results of the exposure to the tested compounds of *I. galbana*. SiNC-DCOIT was the least toxic compound to this species (IC_{50} = 37.4 mg SiNC-DCOIT/L), and was classified as harmful, and free DCOIT was the chemical that presented higher toxicity (IC_{50} = 0.032 mg DCOIT/L), being classified as extremely toxic (Table 2.12). The encapsulation forms of both biocides (SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag) were less toxic than their free form (Table 2.13). Free DCOIT was 214 and 44 times more toxic than in the forms of SiNC-DCOIT and SiNC-DCOIT-Ag, respectively. This reduction of toxicity led DCOIT to change from the extremely toxic category (free form) to the toxic category in both encapsulated forms. The encapsulation of silver in SiNC-Ag and SiNC-DCOIT-Ag reduced its toxicity 2 and 7 times, respectively. In this case, there was only a change in the toxicity category for SiNC-DCOIT-Ag, from very toxic to toxic. In the case of SiNC-Ag, silver remained as very toxic. Silica nanocapsules were also less toxic when combined with the biocides, comparing to the “emply” form.

Table 2.4 – No observed effect concentration (NOEC), lowest observed effect concentration (LOEC) and medium inhibition concentration values (IC_{50}) for the microalgae *Isochrysis galbana* during an exposure of 72 h to SiNC, DCOIT, Ag, SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag.

Contaminant	Units	IC_{50}	95% CI	NOEC	LOEC	Statistics
SiNC	mg SiNC/L	2.10	1.34 – 3.29	1.00	3.00	F=43.7; p=<0.001
DCOIT	mg DCOIT/L	0.032	0.023 – 0.046	0.027	0.081	H=19.8; p=0.001
SiNC-DCOIT	mg DCOIT/L	6.84	4.35 – 10.8	1.00	3.00	F=14.7; p=<0.001
	mg SiNC/L	30.5	19.4 – 48.1	4.46	13.4	
	mg SiNC-DCOIT/L	37.4	23.8 – 58.8	5.46	16.4	
Ag^+	mg Ag^+/L	0.183	0.125 – 0.267	< 0.019	0.019	F=46.8; p=<0.001
SiNC-Ag	mg Ag^+/L	0.420	0.305 – 0.579	0.012	0.036	F=33.1; p=<0.001
	mg SiNC/L	2.46	1.79 – 3.40	0.071	0.213	
	mg SiNC-Ag/L	3.12	2.27 – 4.30	0.090	0.269	
SiNC-DCOIT-Ag	mg SiNC/L	6.33	4.79 – 8.36	< 1.18	1.18	F=47.5; p=<0.001
	mg DCOIT/L	1.42	1.07 – 1.87	< 0.263	0.263	
	mg Ag^+/L	1.31	0.991 – 1.73	< 0.243	0.243	
	mg SiNC-DCOIT-Ag/L	9.84	7.44 – 13.0	< 1.83	1.83	

Microalgae – *Nannochloropsis gaditana*

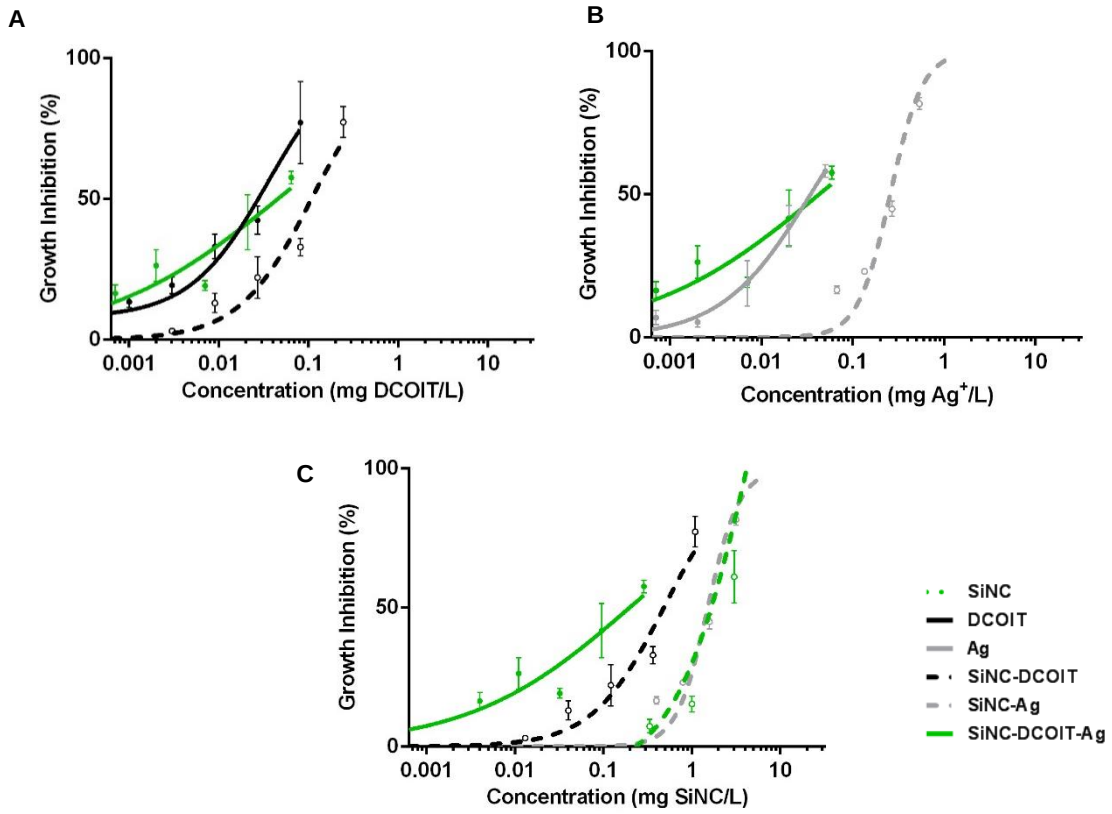


Figure 2.5 – Dose-response curves of *Nannochloropsis gaditana* growth inhibition exposed to: (A) – DCOIT, SiNC-DCOIT and SiNC-DCOIT-Ag (both expressed as DCOIT content); (B) Ag, SiNC-Ag and SiNC-DCOIT-Ag (both expressed as Ag content); (C) SiNC, SiNC-DCOIT, SiNC-Ag, SiNC-DCOIT-Ag (expressed as SiNC content). Data are presented as percentage mean values ± standard error.

Figure 2.5 and Table 2.5 show the results of the exposure of *N. gaditana* to the tested compounds. SiNC-Ag was the least toxic compound to this species (IC_{50} = 1.92 mg SiNC-Ag/L) and free Ag^+ was the chemical that presented higher toxicity (IC_{50} = 0.034 mg Ag^+/L) and both were classified as toxic and extremely toxic, respectively (Table 2.12). The encapsulation of biocides reduced their toxicity in all the tested nanomaterials (SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag) (Table 2.13). The free form of DCOIT was 3.1 and 1.3 times more toxic than in SiNC-DCOIT and SiNC-DCOIT-Ag, respectively. The encapsulation of silver in SiNC-Ag and SiNC-DCOIT-Ag reduced its toxicity 7.6 and 1.3 times. Both DCOIT and silver remained extremely toxic in the SiNC-DCOIT-Ag form, as in their free form, but became toxic in the forms SiNC-DCOIT and SiNC-Ag. Comparing to the "empty form", silica nanocapsules presented higher toxicity when loaded with DCOIT and with the two biocides (DCOIT and Ag).

Table 2.5 – No observed effect concentration (NOEC), lowest observed effect concentration (LOEC) and medium inhitibition concentration values (IC_{50}) for the microalgae *Nannochloropsis gaditana* during an exposure of 72 h to SiNC, DCOIT, Ag, SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag.

Contaminant	Units	IC_{50}	95% CI	NOEC	LOEC	Statistics
SiNC	mg SiNC/L	1.15	0.248 – 5.31	3	9	H =21.6; p=<0.001
DCOIT	mg DCOIT/L	0.035	0.009 – 0.130	0.009	0.027	H=20.7; p=<0.001
SiNC-DCOIT	mg DCOIT/L	0.108	0.083 – 0.140	0.009	0.027	F=45.9; p=<0.001
	mg SiNC/L	0.481	0.370 – 0.626	0.04	0.121	
	mg SiNC-DCOIT/L	0.590	0.452 – 0.766	0.049	0.148	
Ag^+	mg Ag^+/L	0.034	0.026 – 0.046	0.007	0.02	H=20.2; p=0.001
SiNC-Ag	mg Ag^+/L	0.257	0.195 – 0.338	0.268	0.535	H=22.5; p=<0.001
	mg SiNC/L	1.51	1.15 – 1.99	1.58	3.16	
	mg SiNC-Ag/L	1.92	1.45 – 2.53	2.00	3.99	
SiNC-DCOIT-Ag	mg SiNC/L	0.198	0.102 – 0.384	0.032	0.095	H=18.8; p=0.002
	mg DCOIT/L	0.046	0.022 – 0.094	0.007	0.021	
	mg Ag^+/L	0.043	0.021 – 0.088	0.007	0.02	
	mg SiNC-DCOIT-Ag/L	0.316	0.154 – 0.649	0.049	0.146	

Rotifer – *Brachionus plicatilis*

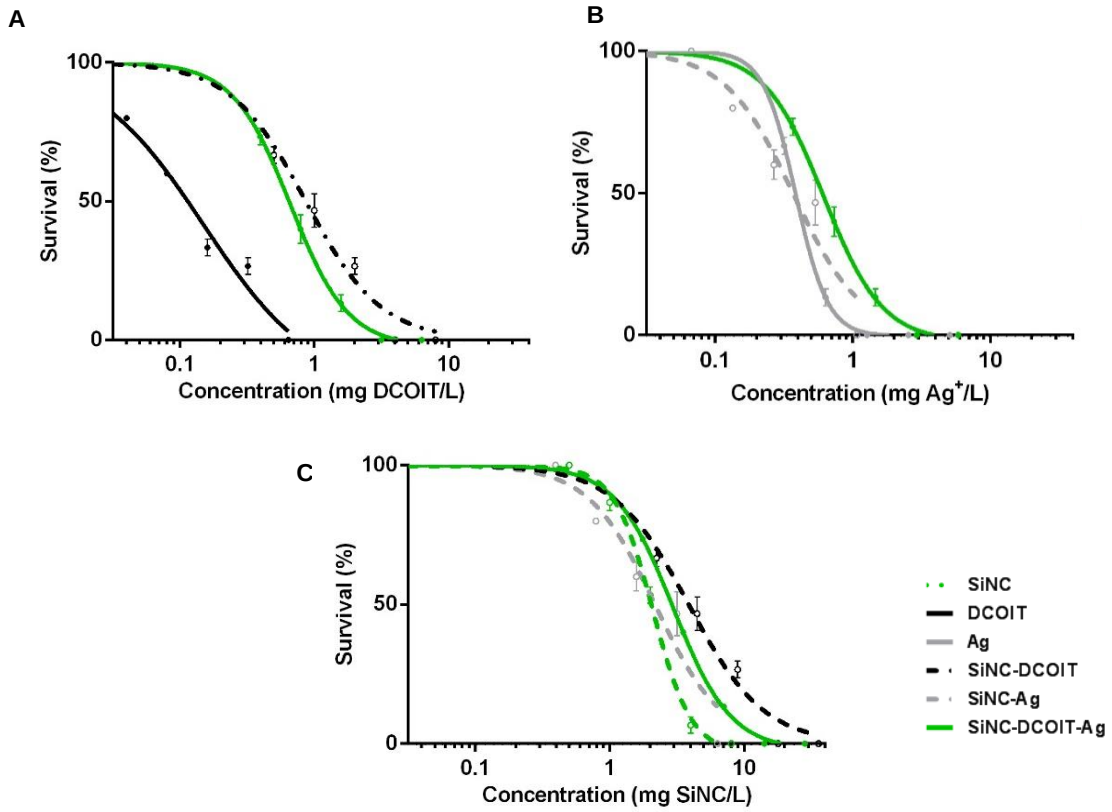


Figure 2.6 – Dose-response curves of *Brachionus plicatilis* survival exposed to: (A) – DCOIT, SiNC-DCOIT and SiNC-DCOIT-Ag (both expressed as DCOIT content); (B) Ag, SiNC-Ag and SiNC-DCOIT-Ag (both expressed as Ag content); (C) SiNC, SiNC-DCOIT, SiNC-Ag, SiNC-DCOIT-Ag (expressed as SiNC content). Data are presented as percentage mean values ± standard error.

Figure 2.6 and Table 2.6 show the results of the exposure of *B. plicatilis* to the tested compounds. SiNC-DCOIT was the least toxic compound to this species (LC_{50} = 4.72 mg SiNC-DCOIT/L), but still globally classified as toxic, and free DCOIT was the chemical that presented higher toxicity, classified as very toxic (LC_{50} = 0.150 mg DCOIT/L) (Table 2.12). The encapsulation of DCOIT reduced its toxicity in both nanomaterials (5.8-fold in SiNC-DCOIT and 4.4-fold in SiNC-DCOIT-Ag) (Table 2.13) but did not change its toxicity category, remaining very toxic. Comparing with the free form, silver was slightly more toxic in the simple nanomaterial (SiNC-Ag) but 1.6-fold less toxic in the nanomaterial containing the two active compounds (SiNC-DCOIT-Ag). As DCOIT, silver also maintained the toxicity category (very toxic) in both encapsulated forms. The sigmoidal curves also show that the toxicity of these three forms of Ag^+ was very similar. Silica nanocapsules were slightly less toxic when loaded with biocides, comparing with their empty form.

Table 2.6 – No observed effect concentration (NOEC), lowest observed effect concentration (LOEC) and medium lethal concentration values (LC_{50}) for the rotifer *Brachionus plicatilis* during an exposure of 24 h to SiNC, DCOIT, Ag, SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag.

Contaminant	Units	LC_{50}	95% CI	NOEC	LOEC	Statistics
SiNC	mg SiNC/L	2.08	1.78 – 2.44	4.00	8.00	H=16.2; p =0.006
DCOIT	mg DCOIT/L	0.150	0.066 – 0.342	0.320	0.640	H=16.6; p=0.005
SiNC-DCOIT	mg DCOIT/L	0.864	0.679 – 1.10	< 0.500	0.5	F = 41.2; p=<0.001
	mg SiNC/L	3.86	3.03 – 4.91	< 2.23	2.23	
	mg SiNC-DCOIT/L	4.72	3.71 – 6.01	< 2.73	2.73	
Ag^+	mg Ag^+/L	0.384	0.346 – 0.428	0.635	1.27	H=15.9; p=0.007
SiNC-Ag	mg Ag^+/L	0.368	0.274 – 0.494	0.535	1.07	H=15.9; p =0.007
	mg SiNC/L	2.17	1.61 – 2.92	3.16	6.31	
	mg SiNC-Ag/L	2.75	2.04 – 3.69	3.99	7.99	
SiNC-DCOIT-Ag	mg SiNC/L	2.96	2.28 – 3.86	7.05	14.1	H=16.0; p=0.007
	mg DCOIT/L	0.665	0.511 – 0.865	1.58	3.16	
	mg Ag^+/L	0.616	0.473 – 0.801	1.46	2.93	
	mg SiNC-DCOIT-Ag/L	4.62	3.55 – 6.01	11.0	21.9	

Bivalve – *Cerastoderma edule*

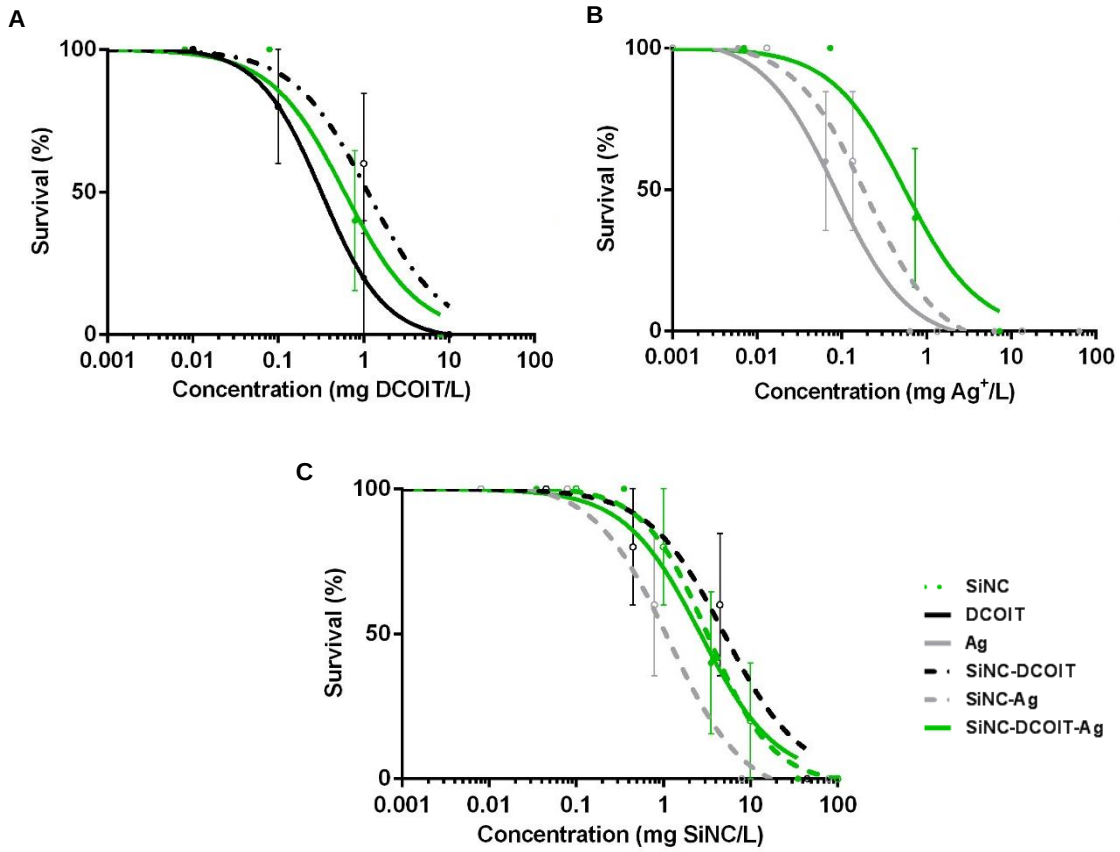


Figure 2.7 – Dose-response curves of *Cerastoderma edule* survival exposed to: (A) – DCOIT, SiNC-DCOIT and SiNC-DCOIT-Ag (both expressed as DCOIT content); (B) Ag, SiNC-Ag and SiNC-DCOIT-Ag (both expressed as Ag content); (C) SiNC, SiNC-DCOIT, SiNC-Ag, SiNC-DCOIT-Ag (expressed as SiNC content). Data are presented as percentage mean values ± standard error.

Figure 2.7 and Table 2.7 show the results of the exposure of *C. edule* to the tested compounds. SiNC-DCOIT was the least toxic compound to this species (LC_{50} = 6.12 mg SiNC/L) and Ag^+ was the chemical that presented higher toxicity (LC_{50} = 0.85 mg Ag^+/L) and both can be classified as toxic and extremely toxic, respectively (Table 2.12). The three novel nanomaterials (SiNC-DCOIT, SiNC-Ag and SINC-DCOIT-Ag) were less toxic than both free biocides (DCOIT and silver) (Table 2.13). DCOIT was 3.5-fold more toxic than its encapsulated form SiNC-DCOIT (decreasing from very toxic to toxic) and also slightly more toxic than in the form SiNC-DCOIT-Ag (but still very toxic in this case, as in free form). Regarding silver, the toxicity was particularly reduced in the form SiNC-DCOIT-Ag, in which it was 6.6-fold less toxic than the free biocide. In SiNC-Ag, Ag^+ presented a toxicity 2.3-fold lower than in its free form. In both encapsulated forms, the toxicity category of silver decreased from extremely toxic to very toxic. Silica nanocapsules had a similar toxicity in all forms.

Table 2.7 – No observed effect concentration (NOEC), lowest observed effect concentration (LOEC) and medium lethal concentration values (LC_{50}) for the bivalve *Cerastoderma edule* during an exposure of 96 h to SiNC, DCOIT, Ag, SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag.

Contaminant	Units	LC_{50}	95% CI	NOEC	LOEC	Statistics
SiNC	mg SiNC/L	3.25	0.850 – 12.4	10.0	100.0	H=17.6; p=0.001
DCOIT	mg DCOIT/L	0.325	0.085 – 1.24	1.00	10.0	H=16.2; p=0.003
SiNC-DCOIT	mg DCOIT/L	1.12	0.411 – 3.05	1.00	10.0	H=15.2; p=0.004
	mg SiNC/L	5.00	1.84 – 13.6	4.47	44.6	
	mg SiNC-DCOIT/L	6.12	2.25 – 16.6	5.47	54.6	
Ag^+	mg Ag^+/L	0.085	0.035 – 0.203	0.064	0.635	H=24.3; p=<0.001
SiNC-Ag	mg Ag^+/L	0.196	0.080 – 0.476	0.134	1.34	H=24.2; p=<0.001
	mg SiNC/L	1.15	0.471 – 2.81	0.789	7.89	
	mg SiNC-Ag/L	1.46	0.598 – 3.56	1.00	10.0	
SiNC-DCOIT-Ag	mg SiNC/L	2.67	1.22 – 5.84	3.52	35.2	H=18.7; p=<0.001
	mg DCOIT/L	0.598	0.273 – 1.31	0.789	7.89	
	mg Ag^+/L	0.554	0.253 – 1.21	0.732	7.32	
	mg SiNC-DCOIT-Ag/L	4.15	2.05 – 8.40	5.48	54.8	

Polychaete – *Hediste diversicolor*

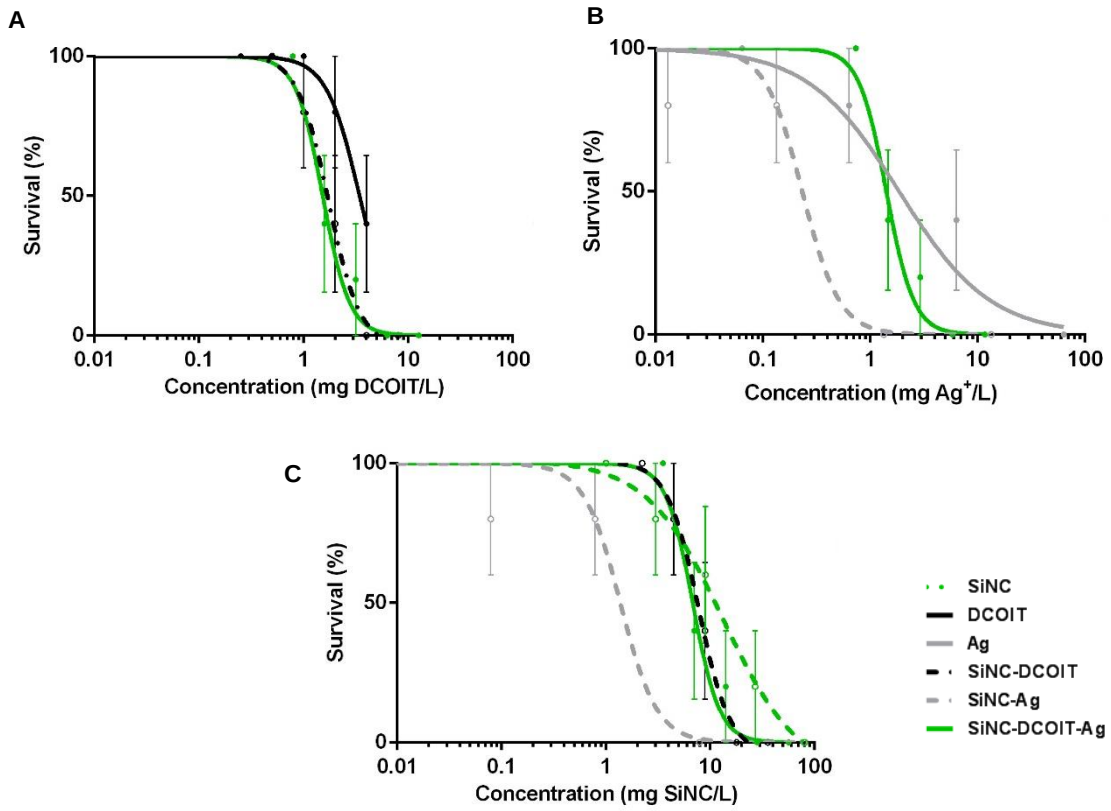


Figure 2.8 – Dose-response curves of *Hediste diversicolor* survival exposed to: (A) – DCOIT, SiNC-DCOIT and SiNC-DCOIT-Ag (both expressed as DCOIT content); (B) Ag, SiNC-Ag and SiNC-DCOIT-Ag (both expressed as Ag content); (C) SiNC, SiNC-DCOIT, SiNC-Ag, SiNC-DCOIT-Ag (expressed as SiNC content). Data are presented as percentage mean values ± standard error.

Figure 2.8 and Table 2.8 show the results of the exposure of *H. diversicolor* to the tested compounds. SiNC was the least toxic compound to this species (LC_{50} = 13.2 mg SiNC/L) and classified as harmful, while SiNC-Ag was the chemical that presented higher toxicity (LC_{50} = 1.73 mg SiNC-Ag/L), globally classified as very toxic (Table 2.12). The encapsulation of both biocides in silica nanocapsules (SiNC-DCOIT, SiNC-Ag and SINC-DCOIT-Ag) did not reduce their toxicity (Table 2.13). Free DCOIT was 2-fold less toxic than when encapsulated in silica nanocapsules alone and 2.3-fold less toxic than SiNC-DCOIT-Ag. Despite this increase, the toxicity category did not change and DCOIT was classified as toxic both in free and encapsulated forms. Free silver was 8 and 1.3-fold less toxic than SiNC-Ag and SiNC-DCOIT-Ag, respectively. In case of SiNC-DCOIT-Ag, silver continued in the toxic category but in case of SiNC-Ag, silver became very toxic. Silica nanocapsules were also more toxic when loaded with the active compounds, comparing with the “empty” capsules.

Table 2.8 – No observed effect concentration (NOEC), lowest observed effect concentration (LOEC) and medium lethal concentration values (LC_{50}) for the polychaete *Hediste diversicolor* during an exposure of 96 h to SiNC, DCOIT, Ag, SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag.

Contaminant	Units	LC_{50}	95% CI	NOEC	LOEC	Statistics
SiNC	mg SiNC/L	13.2	2.70 – 64.4	27.0	81.0	H=17.7; p=0.003
DCOIT	mg DCOIT/L	3.43	0.397 – 0.673	-	-	H=12.3; p=0.031
SiNC-DCOIT	mg DCOIT/L	1.73	1.14 – 2.62	2.00	4.00	H=21.2; p=<0.001
	mg SiNC/L	7.72	5.10 – 11.7	8.93	17.9	
	mg SiNC-DCOIT/L	9.45	6.25 – 14.3	10.9	21.9	
Ag^+	mg Ag^+/L	1.86	0.575 – 5.99	-	-	H=14.6; p=0.005
SiNC-Ag	mg Ag^+/L	0.232	0.029 – 1.89	-	-	H=17.8; p=0.001
	mg SiNC/L	1.38	0.185 – 10.2	-	-	
	mg SiNC-Ag/L	1.73	0.234 – 13.0	-	-	
SiNC-DCOIT-Ag	mg SiNC/L	6.80	5.17 – 8.95	14.1	28.2	H=21.1; p=<0.001
	mg DCOIT/L	1.52	1.15 – 2.00	3.16	6.31	
	mg Ag^+/L	1.41	1.07 – 1.86	1.46	2.93	
	mg SiNC-DCOIT-Ag/L	10.6	8.04 – 13.9	21.9	43.8	

Crustacean – *Artemia salina*

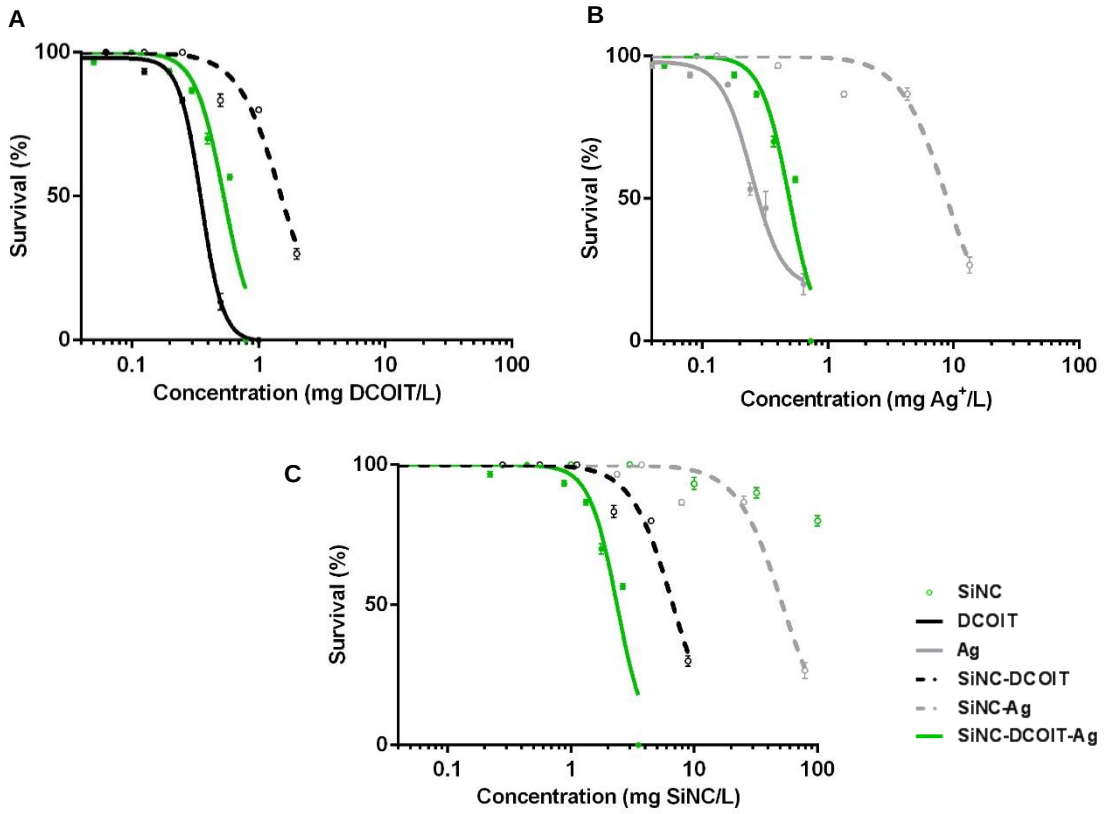


Figure 2.9 – Dose-response curves of *Artemia salina* survival exposed to: (A) – DCOIT, SiNC-DCOIT and SiNC-DCOIT-Ag (both expressed as DCOIT content); (B) Ag, SiNC-Ag and SiNC-DCOIT-Ag (both expressed as Ag content); (C) SiNC, SiNC-DCOIT, SiNC-Ag, SiNC-DCOIT-Ag (expressed as SiNC content). Data are presented as percentage mean values ± standard error.

Figure 2.9 and Table 2.9 show the results of the exposure of *A. salina* to the tested compounds. SiNC was the least toxic compound to this species (LC_{50} > 100 mg SiNC/L) and classified as non-toxic, while free Ag^+ was the chemical that presented higher toxicity (LC_{50} = 0.248 mg Ag^+/L) and was classified as very toxic (Table 2.12). The encapsulation of both biocides in silica nanocapsules (SiNC-DCOIT, SiNC-Ag and SINC-DCOIT-Ag) reduced their toxicity (Table 2.13). The single encapsulation in silica nanocapsules (SiNC-DCOIT and SiNC-Ag) reduced the toxicity of DCOIT and silver in 4.3 and 35.6 times, respectively. In both cases, the two biocides went from very toxic to toxic. Regarding the nanomaterial containing the two biocides (SiNC-DCOIT-Ag), the toxicity reduction was not so marked, but was still of approximately 2-fold for both biocides. In this case, the toxicity category did not change, continuing both biocides as very toxic, as in their free forms. “Empty” silica nanocapsules were less toxic than when loaded with the active compounds.

Table 2.9 – No observed effect concentration (NOEC), lowest observed effect concentration (LOEC) and medium lethal concentration values (LC_{50}) for the crustacean *Artemia salina* during an exposure of 24 h to SiNC, DCOIT, Ag, SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag.

Contaminant	Units	LC_{50}	95% CI	NOEC	LOEC	Statistics
SiNC	mg SiNC/L	> 100	-	-	-	H=11.0; p=0.051
DCOIT	mg DCOIT/L	0.351	0.308 – 0.398	0.500	1.00	H=16.0; p=0.007
SiNC-DCOIT	mg DCOIT/L	1.51	1.34 – 1.69	1.00	2.00	H=19.5; p=0.003
	mg SiNC/L	6.72	6.00 – 7.54	4.46	8.93	
	mg SiNC-DCOIT/L	8.23	7.34 – 9.22	5.46	10.9	
Ag^+	mg Ag^+/L	0.248	0.186 – 0.329	0.16	0.24	F=28.6; p=<0.001
SiNC-Ag	mg Ag^+/L	8.82	7.20 – 10.8	4.28	13.4	F=32.9; p=<0.001
	mg SiNC/L	51.9	42.5 – 63.8	25.3	79.9	
	mg SiNC-Ag/L	65.8	53.8 – 80.8	31.9	100	
SiNC-DCOIT-Ag	mg SiNC/L	2.37	2.15 – 2.61	0.881	1.32	F=120.1; p=<0.001
	mg DCOIT/L	0.531	0.482 – 0.584	0.198	0.296	
	mg Ag^+/L	0.493	0.448 – 0.543	0.180	0.270	
	mg SiNC-DCOIT-Ag/L	3.69	3.35 – 4.06	1.38	2.06	

Crustacean – *Palaemon varians*

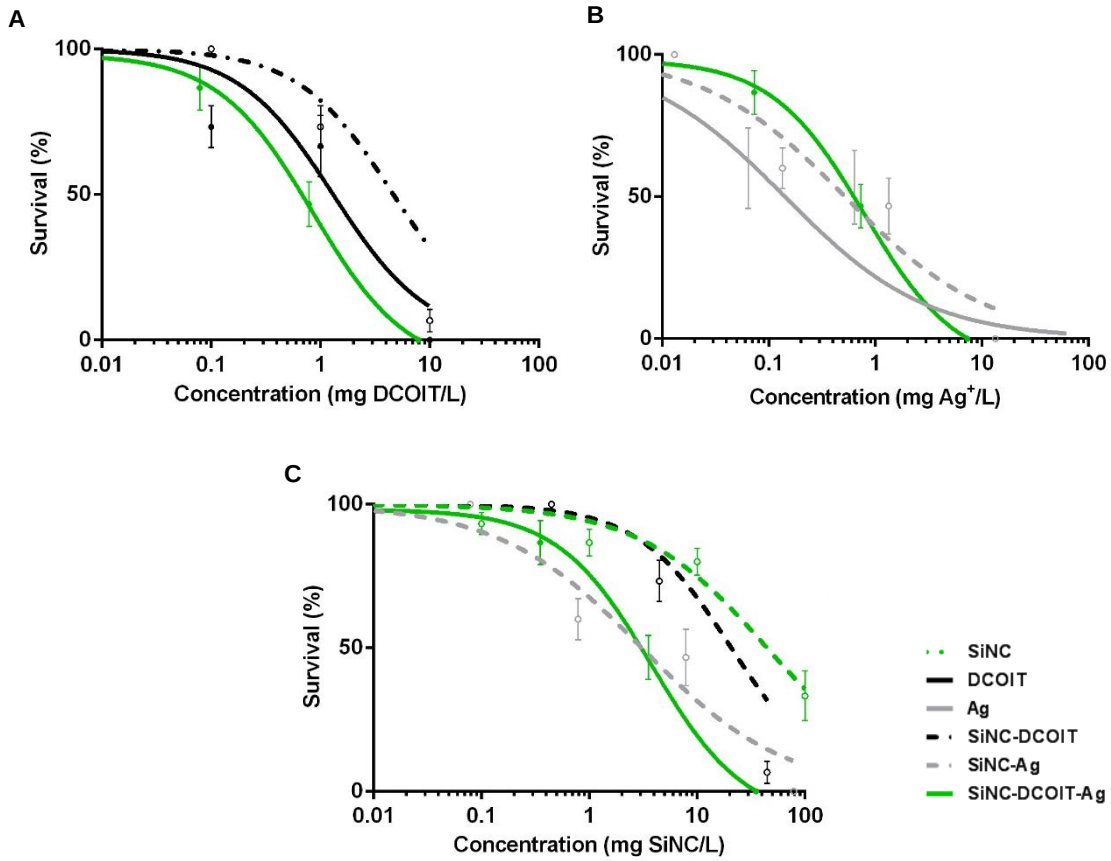


Figure 2.10 – Dose-response curves of *Palaemon varians* survival exposed to: (A) – DCOIT, SiNC-DCOIT and SiNC-DCOIT-Ag (both expressed as DCOIT content); (B) Ag, SiNC-Ag and SiNC-DCOIT-Ag (both expressed as Ag content); (C) SiNC, SiNC-DCOIT, SiNC-Ag, SiNC-DCOIT-Ag (expressed as SiNC content). Data are presented as percentage mean values ± standard error.

Figure 2.10 and Table 2.10 show the results of the exposure to the tested compounds of *P. varians*. SiNC was the least toxic compound to this species (LC_{50} = 44.7 mg SiNC/L) and free Ag^+ was the chemical that presented higher toxicity (LC_{50} = 0.142 mg Ag^+/L), both being classified as harmful and very toxic, respectively (Table 2.12). The encapsulation of DCOIT in the form SiNC-DCOIT reduced 3.6-fold the toxicity of this biocide (Table 2.13), but the same remained classified as toxic. However, in the nanomaterial containing the two biocides (SiNC-DCOIT-Ag), DCOIT was more toxic than in its free form and even the toxicity category changed from toxic to very toxic. The toxicity of silver was lower in both encapsulated forms (3.7-fold in SiNC-Ag and 5.5-fold in SiNC-DCOIT-Ag) but its toxicity category did not change, remaining very toxic as in its free form. Silica nanocapsules showed higher toxicity when loaded with the biocides, comparing with their "empty" form.

Table 2.10 – No observed effect concentration (NOEC), lowest observed effect concentration (LOEC) and medium lethal concentration values (LC_{50}) for the crustacean *Palaemon varians* during an exposure of 96 h to SiNC, DCOIT, Ag, SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag.

Contaminant	Units	LC_{50}	95% CI	NOEC	LOEC	Statistics
SiNC	mg SiNC/L	44.7	19.6 – 101.9	10.0	100.0	F=8.75; p=<0.001
DCOIT	mg DCOIT/L	1.31	0.539 – 3.19	1.00	10.0	H=13.1; p=0.004
SiNC-DCOIT	mg DCOIT/L	4.67	1.86 – 11.7	10.0	> 10.0	H=11.9; p=<0.001
	mg SiNC/L	20.8	8.31 – 52.3	44.6	> 44.6	
	mg SiNC-DCOIT/L	25.5	10.2 – 64.0	54.6	> 54.6	
Ag^+	mg Ag^+/L	0.142	0.034 – 0.586	0.635	6.35	H=15.3; p=0.004
SiNC-Ag	mg Ag^+/L	0.520	0.203 – 1.33	1.34	13.4	H=19.3; p=<0.001
	mg SiNC/L	3.08	1.20 – 7.87	7.89	78.9	
	mg SiNC-Ag/L	3.90	1.52 – 9.97	10.0	100	
SiNC-DCOIT-Ag	mg SiNC/L	3.80	1.29 – 11.2	3.52	35.2	H=16.2; p=0.003
	mg DCOIT/L	0.851	0.288 – 2.52	0.789	7.89	
	mg Ag^+/L	0.788	0.267 – 2.33	0.731	7.31	
	mg SiNC-DCOIT-Ag/L	5.91	2.00 – 17.5	5.48	54.8	

Echinoderm – *Paracentrotus lividus*

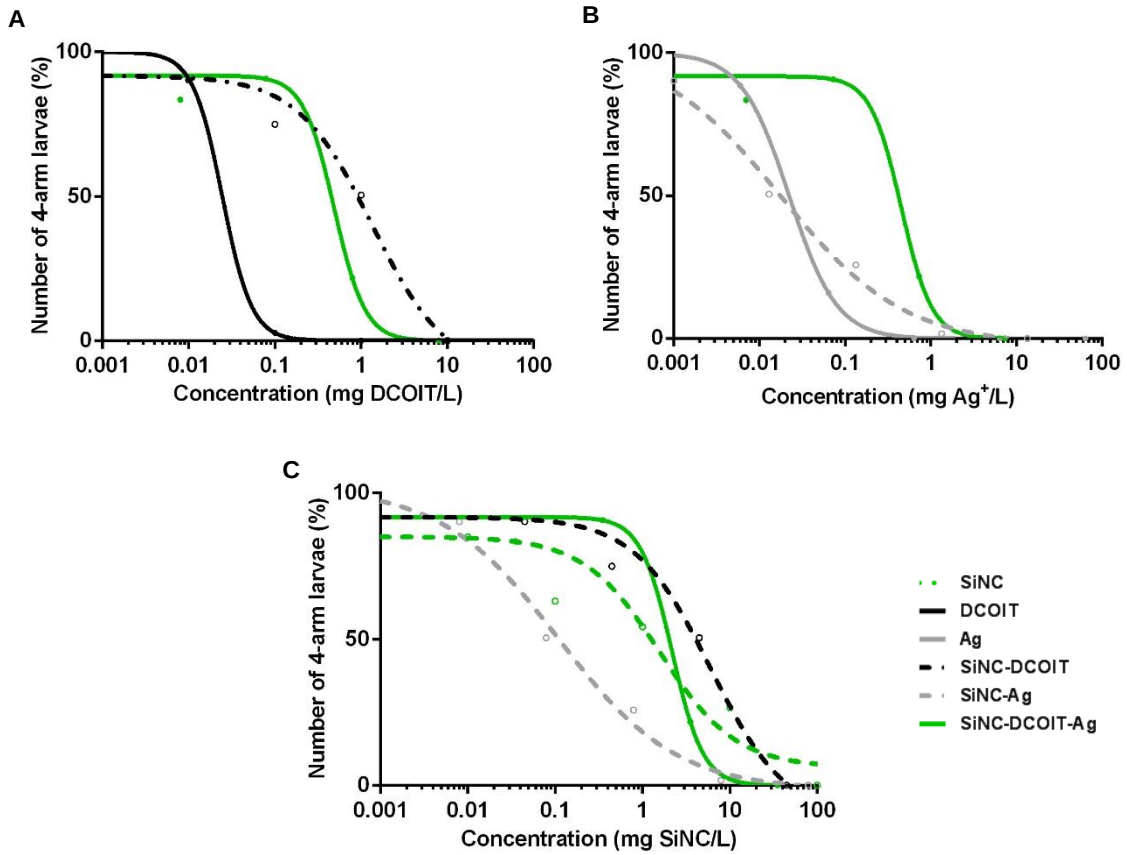


Figure 2.11 – Dose-response curves of *Paracentrotus lividus* number of 4-arm larvae exposed to: (A) – DCOIT, SiNC-DCOIT and SiNC-DCOIT-Ag (both expressed as DCOIT content); (B) Ag, SiNC-Ag and SiNC-DCOIT-Ag (both expressed as Ag content); (C) SiNC, SiNC-DCOIT, SiNC-Ag, SiNC-DCOIT-Ag (expressed as SiNC content). Data are presented as percentage mean values ± standard error.

Figure 2.11 and Table 2.11 show the results of the exposure to the tested compounds of *P. lividus*. SiNC-DCOIT was the least toxic compound to this species (EC_{50} = 7.36 mg SiNC/L) and free Ag^+ was the chemical that presented higher toxicity (EC_{50} = 0.022 mg Ag^+/L), both being classified as toxic and extremely toxic, respectively (Table 2.12). The toxicity of DCOIT was lower in both encapsulated forms (54-fold in SiNC-DCOIT and 19.5-fold in SiNC-DCOIT-Ag) (Table 2.13). Its toxicity category also changed, decreasing from extremely toxic to toxic in SiNC-DCOIT and very toxic in SiNC-DCOIT-Ag. Regarding silver, the free form was 20.5-fold more toxic than the encapsulated form SiNC-DCOIT-Ag, going from extremely toxic to very toxic. However, the silica nanocapsules loaded only with silver (SiNC-Ag) were slightly more toxic than the alone biocide and both forms were classified as extremely toxic. Comparing with the "empty" form, silica nanocapsules showed lower toxicity when loaded with DCOIT and both biocides, but where more toxic when combined only with the bactericidal agent (Ag).

Table 2.11 – No observed effect concentration (NOEC), lowest observed effect concentration (LOEC) and medium effect concentration values (EC_{50}) for the echinoderm *Paracentrotus lividus* during an exposure of 48 h to SiNC, DCOIT, Ag, SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag.

Contaminant	Units	EC_{50}	95% CI	NOEC	LOEC	Statistics
SiNC	mg SiNC/L	1.58	0.725 – 3.44	1.00	10.0	H=23.6; p=<0.001
DCOIT	mg DCOIT/L	0.025	0.023 – 0.027	0.100	1.00	H=18.7; p=<0.001
SiNC-DCOIT	mg DCOIT/L	1.35	0.653 – 1.54	0.100	1.00	H= 18.6; p=<0.001
	mg SiNC/L	6.01	2.91 – 6.89	0.447	4.47	
	mg SiNC-DCOIT/L	7.36	4.91 – 11.0	0.546	5.46	
Ag^+	mg Ag^+/L	0.022	0.020 – 0.025	0.064	0.635	H=22.8; p=<0.001
SiNC-Ag	mg Ag^+/L	0.017	0.013 – 0.024	0.134	1.34	H=22.4; p=<0.001
	mg SiNC/L	0.109	0.079 – 0.152	0.789	7.89	
	mg SiNC-Ag/L	0.138	0.100 – 0.192	1.00	10.0	
SiNC-DCOIT-Ag	mg SiNC/L	2.18	1.19 – 4.00	0.352	3.52	H=18.7; p=<0.001
	mg DCOIT/L	0.488	0.266 – 0.895	0.079	0.789	
	mg Ag^+/L	0.452	0.246 – 0.833	0.073	0.731	
	mg SiNC-DCOIT-Ag/L	3.39	1.71 – 6.73	0.548	5.48	

Table 2.12 – Toxicity categories of SiNC, DCOIT, Ag, SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag for the 11 marine test species used. Classification scheme: **Extremely toxic**: <0.1 mg/L | **Very toxic**: 0.1 - 1 mg/L | **Toxic**: 1 – 10 mg/L | **Harmful**: 10– 100 mg/L | **Non-toxic**: >100 mg/L (EC Directive 93/67/EEC, adapted by Blaise et al. (2008) for ENNs). (T) – target species.

Species	Endpoint	SiNC	DCOIT	Ag	SiNC-DCOIT			SiNC-Ag			SiNC-DCOIT-Ag			
		mg SiNC/L	mg DCOIT/L	mg Ag^+/L	mg SiNC/L	mg DCOIT/L	mg SiNC-DCOIT/L	mg SiNC/L	mg Ag^+/L	mg SiNC-Ag/L	mg SiNC/L	mg DCOIT/L	mg Ag^+/L	mg SiNC-DCOIT-Ag/L
Vibrio fischeri (T)	Inhibition luminescense (IC_{50})													
Phaeodactylum tricornutum (T)	Inhibition growth (IC_{50})													
Mytilus galloprovincialis (T)	Lethality (LC_{50})													
Isochrysis galbana	Inhibition growth (IC_{50})													
Nannochloropsis gaditana	Inhibition growth (IC_{50})													
Brachionus plicatilis	Lethality (LC_{50})													
Cerastoderma edule	Lethality (LC_{50})													
Hediste diversicolor	Lethality (LC_{50})													
Artemia salina	Lethality (LC_{50})													
Palaemon varians	Lethality (LC_{50})													
Paracentrotus lividus	Larval development (EC_{50})													

Table 2.13 – Toxicity ratio (TR) between the L/E/IC_{50} of the free and the encapsulated form of each compound. Bold data highlight cases where biocide encapsulation induced a decrease in toxicity. Each test compound present results based on the concentration of each of the constituents.

Contaminant	Units	*Vibrio fischeri*	*Phaeodactylum tricornutum*	*Mytilus galloprovincialis*	*Isochrysis galbana*	*Nannochloropsis gaditana*	*Brachionus plicatilis*	*Cerastoderma edule*	*Hediste diversicolor*	*Artemia salina*	*Palaemon varians*	*Paracentrotus lividus*
SiNC-DCOIT	mg SiNC/L	0.120	0.014	**8.82**	**14.5**	0.420	**1.86**	**1.54**	0.585	< 1	0.465	**3.81**
	mg DCOIT/L	**1.54**	**1.75**	**30.2**	**214**	**3.09**	**5.76**	**3.45**	0.505	**4.29**	**3.56**	**54.0**
SiNC-Ag	mg SiNC/L	0.046	**1.11**	**1.25**	**1.17**	**1.32**	**1.04**	0.354	0.104	< 1	0.069	0.069
	mg Ag^+/L	0.332	**5.54**	**5.02**	**2.30**	**7.56**	0.958	**2.31**	0.125	**35.6**	**3.66**	0.773
SiNC-DCOIT-Ag	mg SiNC/L	0.024	0.005	**1.01**	**3.01**	0.173	**1.43**	0.822	0.516	< 1	0.085	**1.38**
	mg DCOIT/L	0.311	0.500	**3.35**	**44.4**	**1.32**	**4.43**	**1.84**	0.445	**1.51**	0.650	**19.5**
	mg Ag^+/L	0.217	0.029	**4.83**	**7.16**	**1.26**	**1.60**	**6.52**	0.761	**1.99**	**5.55**	**20.5**

2.5. Discussion

The present findings confirm the very high anti-fouling efficacy of both free biocides towards the target species, namely the bacteria *V. fischeri*, the diatom *P. tricornutum* and the mussel *M. galloprovincialis* (Table 2.12). Bacteria and diatoms are the major groups of organisms firstly adhering to the submerged surfaces during the biofouling process while mussels are one of the last groups of macrofoulers settling. These results are in agreement with past studies in which low concentrations of DCOIT and silver are deleterious for other target organisms, namely diatoms, macroalgae, crustaceans and bivalves (Table 1.1 and 1.3 from Chapter I). An ideal biocide should be effective in preventing the fixation of fouler species and simultaneously should have low toxicity to non-target species, low bioaccumulation in the food web and no persintence in the environment (Jacobson and Willingham, 2000). According to the literature, some booster biocides (like DCOIT) accomplish these characteristics, especially broad-spectrum action and rapid degradation into less toxic compounds (Jacobson et al., 1993; Jacobson and Willingham, 2000; Dafforn et al., 2011). However, some authors refer that their potential action against a wide range of organisms present concern to the environment since they can affect the base of marine food chains and also non-target species at higher levels of the trophic web (Yebra et al., 2004; Dafforn et al., 2011; Price and Readman, 2013). In fact, the present study demonstrated that DCOIT was toxic, very toxic or even extremely toxic towards the non-fouler species (Table 2.12). The extremely toxic effect was verified for the two microalgae species *I. galbana* and *N. gaditana* (as well as for the target species *P. tricornutum*) and for the echinoderm *P. lividus*, indicating that the photosynthetic organisms and the larval stage of the echinoderm were the most sensitive to DCOIT. The extremely toxicity of DCOIT towards these two groups of organisms can be related to the mode of action of this biocide. In the case of *P. lividus*, it can also be explained because a very early and sensitive larval stage of this species was used in the exposure tests, which is in agreement with other studies using the same species (e.g. Bellas, 2006; Bellas, 2007) (Table 1.1 from Chapter I).

Other biocides, such as zinc and copper pyrithiones, Diuron or Irgarol 1051, are also known to induce deleterious effects on non-fouler species as microalgae, copepods, polychaetes, crustaceans, sea urchins, fish, among others, even at low concentrations (e.g. Devilla et al., 2005; Myers et al., 2006; Finnegan et al., 2008; Zhang et al., 2008; Bao et al., 2011) (Table 1.2 from Chapter I). The abovementioned booster biocides were less toxic than DCOIT (comparing each tested species). For the specific case of the echinoderm *P. lividus*, Bellas et al. (2006) estimated an EC_{50} value for Diuron 224-fold

higher than the estimated for DCOIT in the present study. According to the study of Avevelas et al. (2017), zinc and copper pyrithiones are 2.5-fold less toxic comparing with the toxicity of DCOIT recorded in this study towards the diatom *P. tricornutum*. Panagoula et al. (2002) and Koutsaftis and Aoyama (2007) demonstrated that both pyrithiones, Diuron and Irgarol were at least 2.4-fold less toxic towards the crustacean *A. salina*, comparing with DCOIT (this study).

Regarding their comparative fate in the environment, zinc and copper pyrithiones can rapidly degrade in seawater through photolysis, with a reported half-life of less than 24 and 0.5 h, respectively, leaving Zn, Cu and PT^- (ionic pyrithione) as toxic residues. Moreover, both have the potential to persist under reduced light conditions in sediment or deep water (Hellio and Yebra, 2009). In contrast, Irgarol is not easily degraded and has a half-life between 100 and 350 days and Diuron is also considered to be persistent in seawater (Thomas et al., 2002). Although more toxic than these biocides, DCOIT is considered to have low environmental risk comparing to the other existing booster biocides due to its rapid biological degradation in seawater (≤ 24h) (Dafforn et al., 2011).

Silver is a common bactericidal agent (Wijnhoven et al., 2009; Nguyen et al., 2012; Fewtrell, 2014) that demonstrated to be very toxic, or even extremely toxic, towards tested species, presenting a toxicity very close to DCOIT. This is in agreement to other studies (e.g. Jacobson and Willingham, 2000; Braithwaite and Fletcher, 2005; Devilla et al., 2005; Yamada, 2007; Wendt et al., 2016) testing this compound in other marine species (Table 1.3 from Chapter I).

The direct use of biocides in paints may pose several risks for non-target species due to the fast lixiviation from the coatings. Therefore, to minimize this problem and to improve the efficacy of current solutions towards target species, state-of-the-art active compounds were encapsulated/immobilized into smart nanomaterials. This safer-by-design strategy allows the control of the leaching rate of biocides and has already been successfully applied for anti-corrosion purposes through the encapsulation of the corrosion inhibitor MBT in the silica mesoporous nanocapsules (Maia et al., 2012) and in layered double hydroxides (Tedim et al., 2010; Zheludkevich et al. 2012; Martins et al., 2017). In fact, the recent study of Martins et al. (2017) showed that the encapsulation of this corrosion inhibitor in engineered nanoclays (LDH) promotes a decrease of the acute toxicity of MTB to bivalves when immobilized. This technique was also already successfully applied for anti-fouling purposes, through the encapsulation of zinc and copper pyrithiones in LDH or in SiNC (LDH-ZnPT, LDH-CuPT, SiNC-ZnPT and SiNC-CuPT). Avelelas et al. (2017) demonstrated that the encapsulation of biocides reduced their toxicity relatively to their free form against non-target species, without compromise the anti-macrofouling efficacy towards the mussel *Mytilus edulis*. The efficacy of one of

the nanomaterials used in the present study, namely SiNC-DCOIT, was also previously demonstrated in the bacteria *Vibrio fischeri* (Maia et al., 2015).

In line with those findings, the present results also show that the encapsulation of DCOIT and silver in the silica mesoporous nanocapsules (SiNC-DCOIT and SiNC-Ag) generally reduced the toxicity of both biocides for practically all tested species. This reduction was relatively higher in case of SiNC-DCOIT, especially for the non-target species. SiNC-DCOIT exposure was unexpectedly harmful for the target mussel *M. edulis*, which can be explained by the closure of the valves of this organism, a mechanism to avoid exposure to contaminants. Towards the other target tested species, SiNC-DCOIT was still toxic at low concentrations, a very promising result since these species participate in one of the most critical phases of biofouling, the biofilm formation. As expected, SiNC-DCOIT-Ag was globally more toxic than the nanomaterials containing only one active compound, demonstrating that the addition of a silver coating enhances the anti-fouling efficacy of the compound.

Moreover, the smart nanomaterials used for the active compounds encapsulation also reach the marine environment after the biocides release and as coatings end-life, so it is very important that they present no harm to organisms (Avelelas et al., 2017). The present study showed that the unloaded silica nanocapsules were the less toxic tested compound, however, SiNC still was somehow toxic to some species and was more toxic than other nanomaterials used for this purpose (e.g. LDH: Avelelas et al., 2017; Martins et al., 2017; Gutner-Hoch, submitted). The toxicity testing with the surfactant used in the production of SiNC showed that CTAB is globally very toxic to target and non-target species (see supplementary material). Therefore, the removal of this compound after the production process is an extremely important procedure to ensure that these nanocontainers are the least toxic possible to non-target species. However, the presence of traces of CTAB in silica capsules should not be ruled out since a very small quantitiy of this compound may not have been detected due to the detection limits of the equipment used for analysis. Thus, part of the toxicity of SiNC can be due to CTAB.

In conclusion, the encapsulation of biocides in smart ENMs seems to be a promising eco-friendly strategy to develop innovative anti-fouling compounds representing a lower hazard to the environment comparatively to the booster biocides, without compromising their efficacy. However, future studies should address the toxicity of these new compounds in other non-target species and also the bioaccumulation in target and non-target species.

2.6. References

Abarzua, S., and Jakubowski, S. (1995). Biotechnological investigation for the prevention of biofouling. I. Biological and biochemical principles for the prevention of biofouling. M*arine Ecology Progress Series*, 123, 301–312. doi:10.3354/meps123301.

Almeida, E., Diamantino, T., and de Sousa, O. (2007). Marine paints: the particular case of antifouling paints. *Progress in Organic Coatings*, 59(1), 2–20. doi: 10.1016/j.porgcoat.2007.01.017.

Arai, T., Harino, H., Ohji, M., and Langston, W. (2009). Ecotoxicology of Antifouling Biocides. Japan: Springer. ISBN: 978-4-431-85708-2.

ASTM D5660-96 (2009) Standard Test Method for Assessing the Microbial Detoxification of Chemically Contaminated Water and Soil Using a Toxicity Test with a Luminescent Marine Bacterium (Withdrawn 2014). ASTM International, West Conshohocken, PA.

ASTM E729-96 (2002), Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians. ASTM International, West Conshohocken, PA.

ASTM E1440-91 (2012), Standard Guide for Acute Toxicity Test with the Rotifer Brachionus, ASTM International. West Conshohocken, PA.

ASTM E724-98 (2012) Standard guide for conducting static acute toxicity tests starting with embryos of four species of saltwater bivalve molluscs. ASTM International, West Conshohocken, PA.

Avelelas, F., Martins, R., Oliveira, T., Maia, F., Malheiro, E., Soares, A., Loureiro, S., and Tedim, J. (2017). Efficacy and Ecotoxicity of Novel Anti-Fouling Nanomaterials in Target and Non-Target Marine Species. Marine Biotechnology, 19(2), 164–174. doi:10.1007/s10126-017-9740-1.

Bao, V., Leung, K., Qiu, J., and Lam, M. (2011). Acute toxicities of five commonly used antifouling booster biocides to selected subtropical and cosmopolitan marine species. *Marine pollution bulletin*, 62(5), 1147–1151. doi:10.1016/j.marpolbul.2011.02.041.

Bellas, J. (2006). Comparative toxicity of alternative antifouling biocides on embryos and larvae of marine invertebrates. *Science of the Total Environment*, 367(2), 573–585. doi: 10.1016/j.scitotenv.2006.01.028.

Bellas, J. (2007). Toxicity of the booster biocide Sea-Nine to the early developmental stages of the sea urchin Paracentrotus lividus. Aquatic Toxicology, 83(1), 52–61. doi:10.1016/j.aquatox.2007.03.011.

Cao, S., Wang, J., Chen, H., and Chen, D. (2010). Progress of marine biofouling and antifouling technologies. *Chinese Science Bulletin*, 56(7), 598–612. doi:10.1007/s11434-010-4158-4.

Dafforn, K., Lewis, J., and Johnston, E. (2011). Antifouling strategies: History and regulation, ecological impacts and mitigation. *Marine Pollution Bulletin*, 62(3), 453–465. doi:10.1016/j.marpolbul.2011.01.012.

EPA 712–C–96–136 (1996) Mysid Acute Toxicity Test. Environmental Protection Agency, United States.

EPS 1/RM/27 (1992) Biological Test Method: Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars). Environmental Protection Series, Canada.

Férard, J., and Blaise, C. (2013). Encyclopedia of Aquatic Ecotoxicology. Netherlands: Springer. ISBN: 978-94-007-5040-2.

Fewtrell, L. (2014). Silver: water disinfection and toxicity. Aberystwyth University, Aberystwyth.

Gama, B., Pereira, R., and Coutinho, R. (2009). Bioincrustação marinha *in* Pereira, R., Soares-Gomes, A. (2009). Biologia Marinha (2º ed). Rio de Janeiro: Interciência, pp. 299–318.

Gutner-Hoch, E., Martins, R., Oliveira, T., Maia, F., Soares, A., Loureiro, S., Piller, C., Preiss, I., Weis, M., Larroze, S., Teixeira, T., Tedim, J., Benayahu, Y. (Submitted). Antimacrofouling efficacy of innovative inorganic nanomaterials loaded with booster biocides. Journal of Marine Science and Engineering.

Hellio, C., and Yebra, D. (2009). Advances in Marine Antifouling Coatings and Technologies. Boca Raton: CRC Press. ISBN: 978-1-84569-631-3.

Husain, M., and Khan, Z. (2016). Advances in Nanomaterials (Vol. 79). India: Springer. ISBN: 978-81-322-2668-0.

IMO (International Maritime Organization) (2001). International Convention on the Control of Harmful Anti-fouling Systems on Ships.

ISO 11348-3 (2007) Water quality—determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test)–part 3: method using freeze-dried bacteria. International Organization for Standardization.

ISO 10253 (2016) Water quality -- Marine algal growth inhibition test with *Skeletonema* sp. and *Phaeodactylum tricornutum*. International Organization for Standardization.

ISO 19820 (2016) Water quality -- Determination of the acute toxicity to the marine rotifer *Brachionus plicatilis*. International Organization for Standardization.

Jacobson, A., Mazza, L., Lawrence, L., Lawrence, B., Jackson, S., and Kesterson, A. (1993). Fate of an Antifoulant in an Aquatic Environment. *ACS Symposium Series Pesticides in Urban Environments*, 127–138. doi:10.1021/bk-1993-0522.ch012.

Jacobson, A., and Willingham, G. (2000). Sea-nine antifoulant: an environmentally acceptable alternative to organotin antifoulants. *Science of The Total Environment*, 258(1-2), 103–110. doi:10.1016/s0048-9697(00)00511-8.

Koutsaftis, A., and Aoyama, I. (2007). Toxicity of four antifouling biocides and their mixtures on the brine shrimp *Artemia salina*. *Science of the Total Environment*, 387(1), 166–174. doi: 10.1016/j.scitotenv.2007.07.023.

Maia, F., Tedim, J., Lisenkov, A., Salak, A., and Zheludkevich, M. (2012) Silica nanocontainers for active corrosion protection. Nanoscale, 4, 1287–1298. doi:10.1016/j.electacta.2012.04.095.

Maia, F. (2015). Micro/nanoreservoirs for controlled release of active species in smart functional coatings. PhD (Doctor of Philosophy) thesis, University of Aveiro, Portugal.

Maia, F., Silva, A., Fernandes, S., Cunha, A., Almeida, A., Tedim, T., Zheludkevich, M., and Ferreira, M. (2015) Incorporation of biocides in nanocapsules for protective coatings used in maritime applications. Chemical Engineering Journal, 270, 150–157. doi: 10.1016/j.cej.2015.01.076.

Martins, R., Oliveira, T., Santos, C., Kuznetsova, A., Ferreira, V., Avelelas, F., Caetano, A., Tedim, J., Ferreira, M., Freitas, R., Soares, A., and Loureiro, S. (2017). Effects of a novel anticorrosion engineered nanomaterial on the bivalve Ruditapes philippinarum. Environmental Science: Nano, 4(5), 1064–1076. doi: 10.1039/C6EN00630B.

McIntyre, R. (2012). Common nano-materials and their use in real world applications. *Science Progress*, 95(1), 1–22. doi:10.3184/003685012x13294715456431.

Nguyen, T., Roddick, F., and Fan, L. (2012). Biofouling of water treatment membranes: a review of the underlying causes, monitoring techniques and control measures. Membranes, 2(4), 804–840. doi:10.3390/membranes2040804.

OECD (2006) Test no. 201: freshwater alga and cyanobacteria, growth inhibition test. OECD guidelines for the testing of chemicals. Organization for Economic Co-Operation and Development, Paris, Adopted in 23 March 2006, 25 pp.

Price, A., and Readman, J. (2013). Booster biocide antifoulants: is history repeating itself? Late lessons from early warnings: science, precaution, innvation. Part B–Emerging lessons from ecosystems. *The European Environment Agency. Publications office of the European Union, Luxemburg*, Part 12, 297-310.

Readman, J. (2005). Development, Occurrence and Regulation of Antifouling Paint Biocides: Historical Review and Future Trends. *The Handbook of Environmental Chemistry Antifouling Paint Biocides*, 5, 1–15. doi:10.1007/698_5_047.

Tedim, J., Poznyak, S., Kuznetsova, A., Raps, D., Hack, T., Zheludkevich, M., and Ferreira, M. (2010). Enhancement of Active Corrosion Protection via Combination of Inhibitor-Loaded Nanocontainers. *ACS Applied Materials & Interfaces*, 2(5), 1528–1535. doi: 10.1016/j.electacta.2012.04.095.

Thomas, K. (2001). The environmental fate and behaviour of antifouling paint booster biocides: A review. *Biofouling*, 17(1), 73–86. doi:10.1080/08927010109378466.

Yebra, D., Kiil, S., and Dam-Johansen, K. (2004). Antifouling technology – past, present and future steps towards eficiente and environmentally friendly antifouling coatings. *Progress in Organic Coatings*, 50(2), 75–104. doi:10.1016/j.porgcoat.2003.06.001.

WHOI (Woods Hole Oceanographic Institution) (1952). Marine fouling and its prevention - prepared for Bureau of Ships, Navy Deptartment. doi:10.1575/1912/191.

Wijnhoven, S., Peijnenburg, W., Herberts, C., Hagens, W., Oomen, A,, Heugens, E., Roszek, B., Bisschops, J., Gosens, I., van de Meent, D., Dekkers, S., De Jong, W., van Zijverden, M., Sips, A., and Geertsma, R. (2009) Nano-silver – a review of available data and knowledge gaps in human and environmental risk assessment. Nanotoxicology 3(2), 109–138. doi:10.1080/17435390902725914.

Zheludkevich, M., Tedim, J., and Ferreira, M. (2012). “Smart” coatings for active corrosion protection based on multi-functional micro and nanocontainers. Electrochimica Acta, 82, 314–323. doi:10.1016/j.electacta.2012.04.095.

Zhou, F. (2015). Antifouling Surfaces and Materials – From Land to Marine Environment. Berlin: Springer. ISBN: 978-3-662-45204-2.

2.7. Supplementary material

Table 2.1S – Exposure concentration ranges for all chemical substances and all test species.

Test species		***Vibrio fischeri***	***Phaoedactylum tricornutum***	***Mytilus galloprovincialis***	***Isochrysis galbana***	***Nannochloropsis gaditana***	***Brachionus plicatilis***	***Cerastoderma edule***	***Hediste diversicolor***	***Artemia salina***	***Palaemon varians***	***Paracentrotus lividus***
Tested chemicals		**Exposure concentrations range**										
SiNC	mg SiNC/L	0.32 – 81.9	0.01 – 100	0.01 – 100	0.333 – 27	0.333 – 27	0.5 – 8	0.1 – 100	1 – 27	1 – 100	0.1 – 100	0.01 – 100
DCOIT	mg DCOIT/L	0.003 – 0.819	0.001 –0.081	0.01 – 2.43	0.001 – 0.081	0.001 – 0.081	0.04 – 0.64	0.01 – 10	0.25 – 4	0.0625 – 1	0.1 – 10	0.01 – 10
$AgNO_3$	mg Ag^+/L	0.204 – 52.0	0.006 – 0.463	0.006 – 63.5	0.019 – 1.54	0.001 – 0.050	0.318 – 5.08	0.006 – 63.5	0.064 – 63.5	0.040 – 0.640	0.064 – 63.5	0.006 – 63.5
SiNC-DCOIT	mg SiNC/L	0.015 – 3.66	0.004 – 0.362	0.045 – 446	1.49 – 40.2	0.013 – 1.09	2.23 – 35.7	0.045 – 44.6	2.23 – 35.7	0.279 – 8.93	0.446 – 44.6	0.045 – 44.6
	mg DCOIT/L	0.003 – 0.646	0.001 – 0.081	0.01 – 100	0.333 – 9	0.003 – 0.243	0.5 – 8	0.01 – 10	0.5 – 8	0.0625 – 2	0.1 – 10	0.01 – 10
SiNC-Ag	mg SiNC/L	0.014 – 3.53	0.008 – 78.9	0.008 – 78.9	0.071 – 5.75	0.395 – 6.31	0.395 – 6.31	0.008 – 78.9	0.079 – 78.9	3.75 – 78.9	0.079 – 78.9	0.008 – 78.9
	mg Ag^+/L	0.003 – 0.600	0.001 – 13.4	0.001 – 13.4	0.012 – 0.980	0.067 – 1.07	0.067 – 1.07	0.001 – 13.4	0.013 – 13.4	0.211 – 13.4	0.013 – 13.4	0.001 – 13.4
SiNC-DCOIT-Ag	mg SiNC/L	0.012 – 2.89	0.004 – 0.35	0.035 – 352	1.17 – 31.7	0.004 – 0.286	1.75 – 28	0.035 – 35.2	3.5 – 56	0.130 – 3.523	0.352 – 35.2	0.035 – 35.2
	mg DCOIT/L	0.003 – 0.646	0.001 – 0.064	0.008 – 79	0.259 – 7.10	0.001 – 0.064	0.375 – 6	0.008 – 7.89	0.788 – 12.6	0.05 – 0.789	0.079 – 7.89	0.008 – 7.89
	mg Ag^+/L	0.003 – 0.600	0.001 – 0.059	0.007 – 73.1	0.243 – 6.58	0.001 – 0.059	0.366 – 5.85	0.007 – 7.32	0.732 – 11.7	0.050 – 0.730	0.073 – 7.31	0.007 – 7.31
Dilution factor		*2*	*3 (apart from SiNC; CTAB; SiNC-Ag=10)*	*10 (apart from DCOIT=3)*	*3*	*3 (apart from CTAB=10; SiNC-Ag=2)*	*2*	*10*	*2 (apart from $AgNO_3$; SiNC-Ag=10; CTAB=3)*	*2 (apart from SiNC; SiNC-Ag=10)*	*10*	*10*

2.7.1. Toxicity of CTAB

The exposure concentrarion ranges of CTAB used in the ecotoxicity testing for each species are shown in Table 2.2S, as well as the corresponding results. Overall, CTAB was very toxic to the tested species (L/E/IC_{50} values ranging between 0.08 to 3.33 mg CTAB/L). The most and less sensitive species were the microalgae *N. gaditana* and the bivalve *M. galloprovincialis*, respectively. These results show that CTAB induces effects on organisms at low concentrations, so that its removal of the silica nanocapsules (SiNC) after the production process is essential to ensure that the silica capsules are less toxic to non-target organisms.

Table 2.2S – Medium lethal/effect/inhibition concentration values (L/E/IC_{50}) for the tested species during the respective exposure times to CTAB. Toxicity category scheme: **Extremely toxic: <0.1 mg/L** | **Very toxic: 0.1 - 1 mg/L** | **Toxic: 1 – 10 mg/L** | **Harmful**: 10– 100 mg/L | **Non toxic**: >100 mg/L (EC Directive 93/67/EEC, adapted by Blaise et al. (2008) for ENNs). (T) – target species; nt – not tested.

Species	Exposure concentration ranges (mg CTAB/L)	L/E/IC_{50}	95% CI	Toxicity category	NOEC	LOEC	Statistics
Vibrio fischeri (T)	nt	nt	nt	nt	nt	nt	nt
Phaeodactylum tricornutum (T)	0.01 – 100	0.963	0.085 – 0.143		-	0.010	F=13,3; p=<0,001
Mytilus galloprovincialis (T)	0.01 – 100	3.33	1.93 – 5.77		1,00	10,0	H=63,2; p=<0,001
Isochrysis galbana	0.09 – 7.29	0.470	0.388 – 0.570		-	0.090	F=130,1; p=<0,001
Nannochloropsis gaditana	0.01 – 100	0.080	0.025 – 0.252		0.010	0.100	F=26,1; p=<0,001
Brachionus plicatilis	0.5 – 8	0.958	0.831 – 1.10		2.00	4.00	H=16,2; p=0,006
Cerastoderma edule	0.01 – 100	0.317	0.110 – 0.916		1.00	10.0	H=22,8; p=<0,001
Hediste diversicolor	0.03 – 2.43	0.468	0.320 – 0.685		0.810	2.43	H=22,0; p=<0,001
Artemia salina	nt	nt	nt	nt	nt	nt	nt
Palaemon varians	0.01 – 100	2.49	1.49 – 4.18		1.00	10.0	H=22,5; p=<0,001
Paracentrotus lividus	0.01 – 100	1.12	0.792 – 1.59		1.00	10.0	H=22,6; p=<0,001

Chapter III

Use of species sensitivity distribution curves to assess the ecological hazard of new anti-fouling nano-based solutions in marine species

3. Use of species sensitivity distribution curves to assess the ecological hazard of new anti-fouling nano-based solutions in marine species

Joana Figueiredo[1], Roberto Martins[2], Tânia Oliveira[3], Frederico Maia[3], João Tedim[4], Amadeu Soares[2] and Susana Loureiro[2]

[1] Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[2] Department of Biology and CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[3] Smallmatek - Small Materials and Technologies, Lda., Rua Canhas, 3810-075 Aveiro, Portugal

[4] Department of Materials and Ceramic Engineering, CICECO, University of Aveiro, 3810-193 Aveiro, Portugal

3.1. Abstract

Biocide-based coatings have been applied for several years to tackle marine biofouling. Recently, engineered nanomaterials, such as silica mesoporous nanocapsules (SiNC), were used to encapsulate biocides in order to control their release to the environment. An example is the encapsulation of the biocides DCOIT and silver in this nanostructure (SiNC-DCOIT and SiNC-Ag), as well as a new modified version including the two biocides (SiNC loaded with DCOIT and coated with silver, SiNC-DCOIT-Ag). This study aims to assess the ecological hazard of these three novel compounds and their free counterparts (DCOIT and silver) to marine ecosystems. For this, marine toxicity data (L/E/IC_{50} or NOEC) was compiled to construct species sensitivity distributions (SSDs) and to calculate the hazardous concentration at 5% (HC_5) in order to derive a PNEC for each test compound. In the different approaches to construct the SSDs, the estimated HC_5 and PNEC values were lower for the encapsulated biocides than for their free form. Therefore, the encapsulation of biocides in engineered nanomaterials appears to be a promising solution to produce new anti-fouling compounds to be incorpored in coatings, with lower environmental hazard than biocides by themselves.

Keywords: Biofouling; DCOIT; Silver; Nanomaterial; Species sensitivity distribution (SSD); Marine; Hazard assessment

3.2. Introduction

Organotin compounds (particularly TBT) have been widely used as anti-fouling agents since the late 1950s (Yebra et al., 2004; Readman, 2005; Hellio and Yebra, 2009). However, since the use of these chemicals was banned in 2008 (IMO, 2001), many new biocides have been developed and used in commercial anti-fouling paints (Thomas, 2001; Yebra et al., 2004; Dafford et al., 2011). DCOIT is one of these new compounds which is a booster biocide that inhibits both soft and hard fouling (Jacobson and Willingham, 200). This active compound is easily biodegraded, with half-life in natural seawater less than 24 hours (Shade et al., 1993; Willingham and Jacobson, 1996; Thomas, 2001). Although presenting a rapid degradation in seawater, this biocide still represents concern for the environment as it has a broad-spectrum action and can affect many non-target organisms from lower to higher trophic levels in the first hours of activity, affecting marine food chains (Yebra et al., 2004; Dafforn et al., 2011; Price and Readman, 2013). Alternative techniques to prevent biofouling have also been developed, such the use of enzymes, biomimetization, electrolysis of seawater, vibration, among others (Bers et al., 2006; Fusetani and Clare, 2006; Hellio and Yebra, 2009; Cao et al., 2010; Salta et al., 2010; Zhou, 2015) but they are still not able to substitute anti-fouling coatings based on biocides. Biocides continue to be considered the most efficient, resistant, easy to apply alternative, with low maintenance and cost-effectiveness. On the other hand, there is an increasing pressure from governments and industry to develop new solutions for products containing biocides maintaining their efficacy but reducing the toxicity in order to make them more environmentally friendly (Almeida et al., 2007). One of these solutions is the use of nanotechnology to encapsulate the active ingredients of coatings, allowing their controlled release and preventing their inactivation and interaction with other paint components (Maia, 2015). This new technique was already successfully applied in anti-corrosion coatings through the encapsulation of corrosion inhibitors in ENMs, namely in silica mesoporous-nanocapsules (SiNC) (Maia et al., 2012) and in layered double hydroxides (LDH) (Tedim et al., 2010; Zheludkevich et al. 2012; Martins et al., 2017). More recently, the same assumption was used to encapsulate DCOIT, zinc pyrithione or copper pyrithione in SiNC (SiNC-DCOIT: Maia et al., 2015; SiNC-ZnPT and SiNC-CuPT: Avelelas et al., 2017) or in LDH (LDH-ZnPT and LDH-Cu-PT: Avelelas et al., 2017) to produce innovative nano-structured anti-fouling additives. Currently, the Portuguese company Smallmatek, Lda. is doing the scale-up of these nanomaterials and testing the possibility of their industrial production. Meanwhile, this company is developing other nanomaterials. In order to enhance the anti-fouling efficacy of the compound Smallmatek proposed an external silver coating for SiNC-DCOIT

(SiNC-DCOIT-Ag). The assumption is that the microbial biofilm formation is one of the first and critical stages of biofouling and silver is known as a bactericidal agent that kills a broad-spectrum of microorganisms (Wijnhoven et al., 2009; Nguyen et al., 2012; Fewtrell 2014).

Although the efficacy of SiNC-DCOIT has already been demonstrated in the bacteria *Vibrio fischeri* (Maia et al., 2015) and the impacts of SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag and their respective counterparts in target and non-target organisms have been assessed (see Chapter I) their potential ecological hazard have not yet been investigated. In the recent past, products containing nanomaterials, as well as other chemical compounds, were developed, produced and released on the market without any study of their impact on the environment, namely their behavior, fate and toxicity to organisms. However, there are currently several directives and regulations, like REACH, CLP among others, to evaluate whether or not a compound can be commercialized with the aim of protecting human and environmental health, and one of the topics required for product approval is the risk assessment. For the specific case of biocides, the regulation 528/2012 (EU) is the one to be fulfilled in the case of anti-fouling compounds using biocides. In this regulation, regarding environmental assessment, it is highlighted that "components in the product may influence the fate and behaviour (and ecotoxicity) of the active substance". In addition, this regulation also refers to REACH and CLP, for the case of mixtures and synergisms. The biocidal products regulation advises to carry out a dose-response approach to predict concentrations where no adverse effects will occur. The predicted no-effect concentrations (PNECs) should be then derived from the available ecotoxicity testing (e.g. EC_{50}, NOEC or LOEC) with adequate assessment factors.

The PNEC is usually derived from the lowest NOEC value in a dataset, with an adequate safety factor, which is dependent on the dataset quality and quantity, within a deterministic approach. Recently a statistical extrapolation methodology has been also used to predict this "no adverse effect" concentration by the calculation of the hazardous concentration (HC) that affect a certain proportion of a set of species (*p*%). This approach includes the construction of species sensitivity distributions (SSDs), which includes the sensitivity variability inherent to different species to an exposure to stressors through a statistical distribution function (Forbes and Callow, 2002; Posthuma et al., 2002; Maltby, 2005; Silva et al., 2014). The estimation of HCs is used to obtain predicted no-effect concentration (PNECs) values, and the HCs are derived to protect ecosystems against the adverse effects of hazardous chemicals (Poshuma et al., 2002). Generally, it is calculated the HC_5 value (hazardous concentration for 5% of species), which means that 95% of the considered species are protected if the concentration of the chemical

compound in the environment is below this value (Aldenberg and Jaworska, 2000; Posthuma et al., 2002; Maltby et al., 2005; Garner et al., 2015). Besides this approach does not evaluate any interaction between species and does not consider the ecosystem functioning and the bioavailability of the chemical compound since the toxicity of the compounds are assessed individually (Aldenberg and Jaworska, 2000; Posthuma et al., 2002; Maltby et al., 2005; Garner et al., 2015), it has the advantage of providing an overview of the likelihood of toxicity for the selected species. Also, if the SSDs are constructed with data for relevant species of different trophic levels and phyla it is possible to have a more accurate picture of the impacts in the environment, which is of extreme importance in the case of compounds for which few to no information is available (Silva et al., 2014).

Although the use of chronic toxicity data is the most appropriate to construct the SSDs (as in any approach for risk assessment), where the magnitude and type of effects are ecologically more relevant, the use of acute toxicity data has some advantages, namely: (a) less laboratory maintenance in acute tests; (b) possibility of use more species; (c) more reliable data found in literature belonging to all major taxonomic groups, due to the existence of more standard protocols; and (d) limited number of responses and time scales (e.g. 96 h mortality), generally easy to interpret. Moreover, the hazard associated with the biocides used in the present study (especially DCOIT) are of short-term, due to their rapid degradation in seawater, which are more in accordance with the duration of already described acute or short-term chronic toxicity tests.

To date, many SSDs have been developed for a wide variety of organic and inorganic pollutants, particularly pesticides and herbicides. Zinc, copper and cadmium are amongst the most studied metals. Most of these studies were done for freshwater ecosystems (Wheeler et al., 2002; Garner et al., 2015), due to the greater abundance of reliable quality data for these organisms, while there is usually less data available for saltwater species, especially for organic compounds (Wheeler et al., 2002). Concerning nanomaterials, SSDs with freshwater organisms have been done for nano-Cu (Adam et al., 2015; Lützhøft et al., 2015), nano-Au (Botha et al., 2015), nano-Ag, nano-TiO_2, nano-ZnO, carbon nanotubes (Lützhøft et al., 2015; Coll et al., 2016), nano-CeO_2 and nano-nZVI (Lützhøft et al., 2015) (Table 3.2S). Regarding marine species, an exhaustive research in the literature was done and studies with metals (Hg, Cu, Cd, Pb, Zn: Durán and Beiras, 2013; Ni: Deforest and Schlekat, 2013), pesticides (cypermethrin, endosulfan, chlorpyrifos and fenvalerate: Bollmohr et al., 2007) and anti-fouling compounds (Tributyltin: Leung et al., 2007 and Schipper et al., 2008; Pyridine Triphenylborane: Mochida et al., 2012; DCOIT: Mochida et al., 2015) were found.

Regarding marine water, PNEC values for multi-walled carbon nanotubes (MWCNT) and carbon black (CB) were found on literature (Table 3.2S), however no SSDs for ENMs (including NPs) with marine species were found.

Therefore, and considering the amount and quality of data generated for different novel anti-fouling nanomaterials (from chapter I), the main goal of this study was to predict the no effect concentration for the single compounds SiNC, DCOIT and Ag and the novel nanomaterials containing one or two biocides (SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag), through the construction of SSDs and through the deterministic PNEC derivation.

3.3. Materials and Methods

3.3.1. Data selection

The L/E/IC_{50} and/or NOEC values obtained in chapter I were used to construct species sensitivity distributions and to calculate the HC_5 and the marine PNEC values for each compound. For SiNC, DCOIT and Ag, toxicity data (L/E/IC_{50} and NOEC) was also gathered from the literature (For L/E/IC_{50} data – DCOIT: Table 1.1; Silver: Table 1.3; SiNC: pp. 19, from chapter I; For NOEC data – Table 3.1S).

3.3.2. Species sensitivity distributions and HC_5 calculation

Species sensitivity distributions (SSDs) and the hazardous concentration at 5% (HC_5) were estimated using a log-normal distribution through the software *ETX* 2.1. Four different approaches were considered in the construction of SSDs: L/E/IC_{50} data grouped by species, NOEC data grouped by species, L/E/IC_{50} data grouped by phylum and NOEC data grouped by phylum. In situations where there were several values for the same species/phylum, the geometric mean of the values was performed. Generating different SSDs grouped taxonomically by species and phylum can provide different outputs as phylum include species using several morphological and functional traits.

The derivation of the PNEC was calculated by the ratio between HC_5 and an assessment factor of 5 (most conservative factor), following the technical guidance document (TGD, 2003).

3.3.3. Deterministic PNEC calculation

The PNEC was determined using the lowest NOEC values for the dataset available for each test compound. In addition, another deterministic exercise was carried out to determine the PNEC based on the lowest L/E/IC_{50} available. The assessment factors used for each approach are shown in Table 3.1.

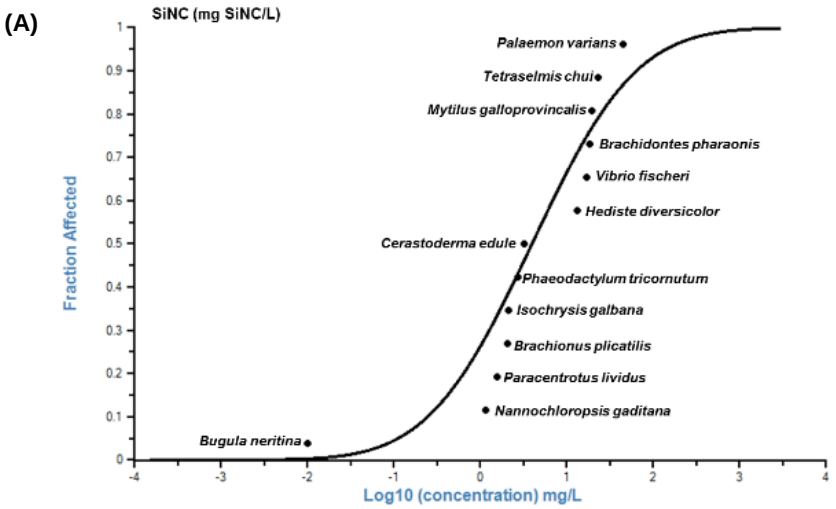
Table 3.1 – Assessment factors used for each compound proposed for deriving $PNEC_{determ}$ for saltwater for different data sets (TGD, 2003).

Compound	Approach	Assessment Factor	Data set
All	L/E/IC_{50} data	1000	Lowest short-term L/E/IC_{50} from freshwater or saltwater representatives of three taxonomic groups of three trophic levels + two additional marine taxonomic groups
All	NOEC data	100	Lowest long-term NOECs from three freshwater or saltwater species three trophic levels*

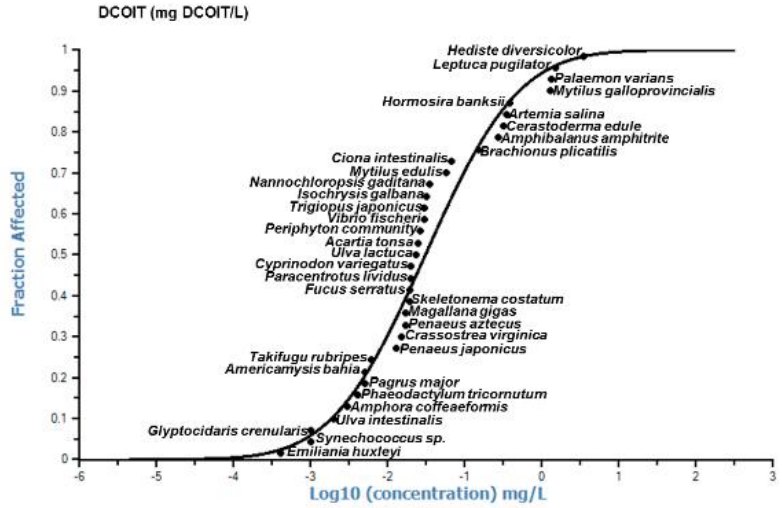
* Reduced assessment factor to 100 if only one short-term test or to 50 if two short-term tests on marine species are available) applied to the lowest NOEC from only two species may be appropriate where short-term tests for additional species representing marine taxonomic groups (for example echinoderms or molluscs) have been carried out and indicate that these are not the most sensitive group

3.4. Results

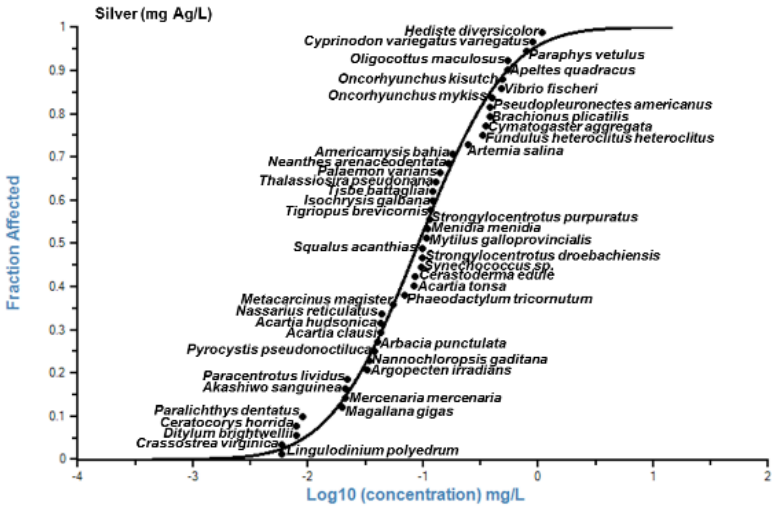
3.4.1. Species sensitivity distributions (SSDs) – data grouped by species



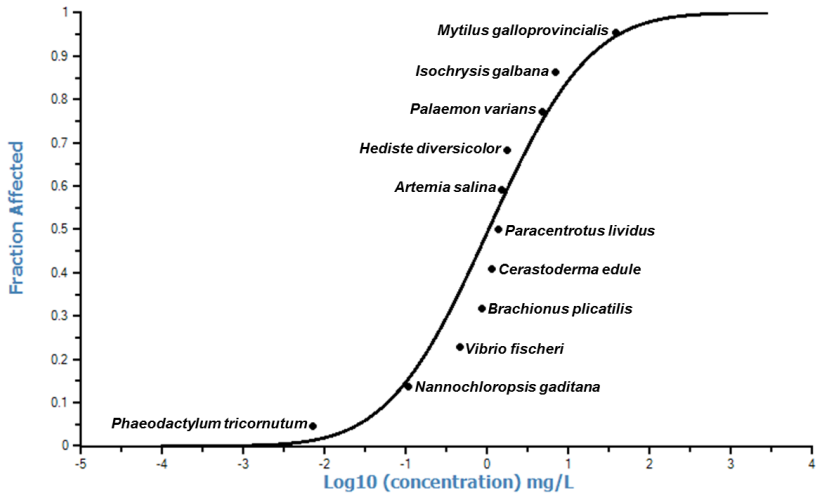
(B)


DCOIT (mg DCOIT/L)
Fraction Affected
Log10 (concentration) mg/L
Hediste diversicolor
Leptuca pugilator
Palaemon varians
Mytilus galloprovincialis
Hormosira banksii
Artemia salina
Cerastoderma edule
Amphibalanus amphitrite
Brachionus plicatilis
Ciona intestinalis
Mytilus edulis
Nannochloropsis gaditana
Isochrysis galbana
Trigiopus japonicus
Vibrio fischeri
Periphyton community
Acartia tonsa
Ulva lactuca
Cyprinodon variegatus
Paracentrotus lividus
Fucus serratus
Skeletonema costatum
Magallana gigas
Penaeus aztecus
Crassostrea virginica
Penaeus japonicus
Takifugu rubripes
Americamysis bahia
Pagrus major
Phaeodactylum tricornutum
Amphora coffeaeformis
Ulva intestinalis
Glyptocidaris crenularis
Synechococcus sp.
Emiliania huxleyi

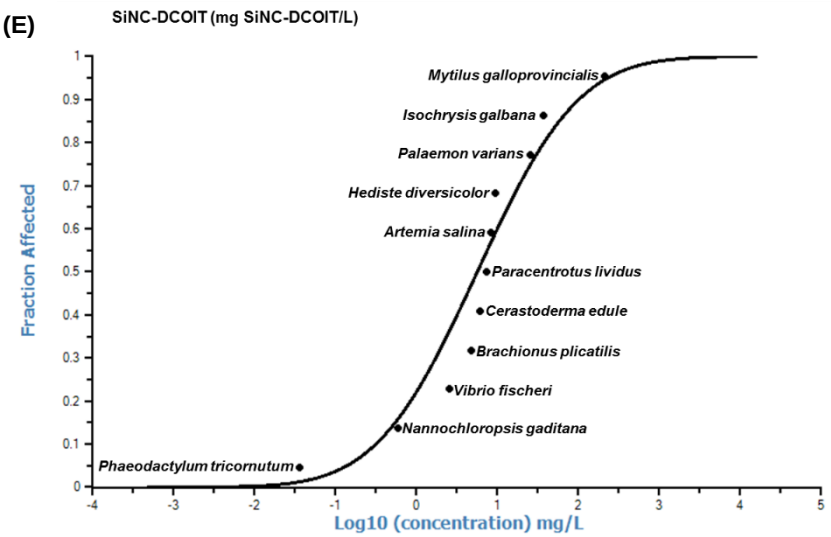
(C)


Silver (mg Ag/L)
Fraction Affected
Log10 (concentration) mg/L
Hediste diversicolor
Cyprinodon variegatus variegatus
Oligocottus maculosus
Paraphys vetulus
Apeltes quadracus
Oncorhynchus kisutch
Vibrio fischeri
Oncorhynchus mykiss
Pseudopleuronectes americanus
Brachionus plicatilis
Cymatogaster aggregata
Fundulus heteroclitus heteroclitus
Americamysis bahia
Artemia salina
Neanthes arenaceodentata
Palaemon varians
Thalassiosira pseudonana
Ysbe battagliae
Isochrysis galbana
Tigriopus brevicornis
Strongylocentrotus purpuratus
Menidia menidia
Mytilus galloprovincialis
Squalus acanthias
Strongylocentrotus droebachiensis
Synechococcus sp.
Cerastoderma edule
Acartia tonsa
Metacarcinus magister
Phaeodactylum tricornutum
Nassarius reticulatus
Acartia hudsonica
Acartia clausi
Arbacia punctulata
Pyrocystis pseudonoctiluca
Nannochloropsis gaditana
Argopecten irradians
Paracentrotus lividus
Akashiwo sanguinea
Mercenaria mercenaria
Paralichthys dentatus
Magallana gigas
Ceratocorys horrida
Ditylum brightwellii
Crassostrea virginica
Lingulodinium polyedrum

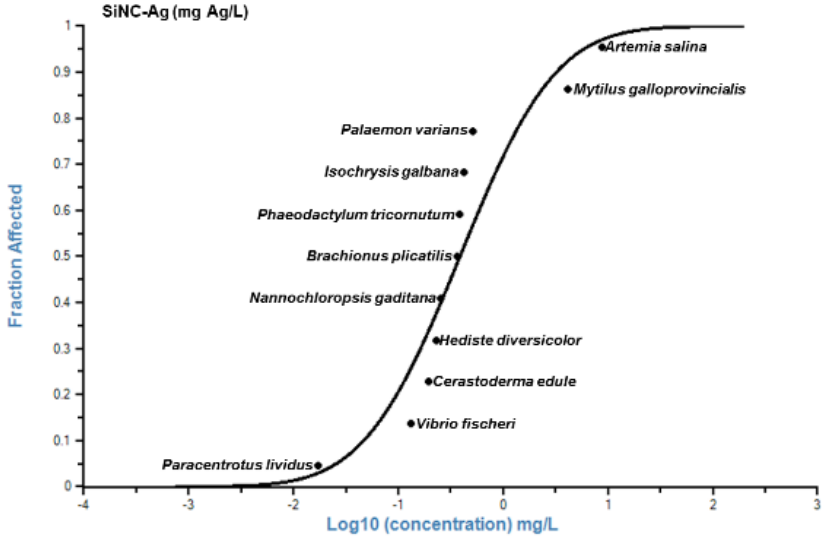
(D)


SiNC-DCOIT (mg DCOIT/L)
Fraction Affected
Log10 (concentration) mg/L
Mytilus galloprovincialis
Isochrysis galbana
Palaemon varians
Hediste diversicolor
Artemia salina
Paracentrotus lividus
Cerastoderma edule
Brachionus plicatilis
Vibrio fischeri
Nannochloropsis gaditana
Phaeodactylum tricornutum

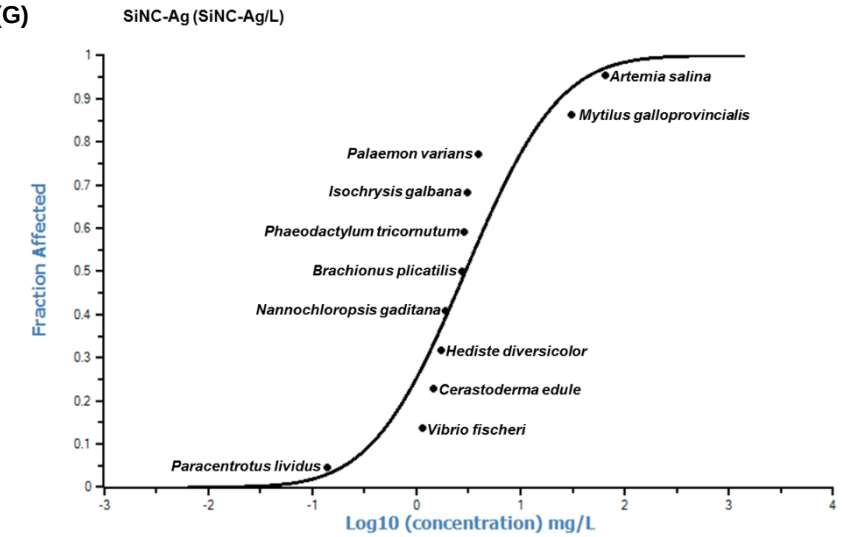
(E)


SiNC-DCOIT (mg SiNC-DCOIT/L)
Fraction Affected
Log10 (concentration) mg/L
Mytilus galloprovincialis
Isochrysis galbana
Palaemon varians
Hediste diversicolor
Artemia salina
Paracentrotus lividus
Cerastoderma edule
Brachionus plicatilis
Vibrio fischeri
Nannochloropsis gaditana
Phaeodactylum tricornutum

(F)


SiNC-Ag (mg Ag/L)
Fraction Affected
Log10 (concentration) mg/L
Artemia salina
Mytilus galloprovincialis
Palaemon varians
Isochrysis galbana
Phaeodactylum tricornutum
Brachionus plicatilis
Nannochloropsis gaditana
Hediste diversicolor
Cerastoderma edule
Vibrio fischeri
Paracentrotus lividus

(G)


SiNC-Ag (SiNC-Ag/L)
Fraction Affected
Log10 (concentration) mg/L
Artemia salina
Mytilus galloprovincialis
Palaemon varians
Isochrysis galbana
Phaeodactylum tricornutum
Brachionus plicatilis
Nannochloropsis gaditana
Hediste diversicolor
Cerastoderma edule
Vibrio fischeri
Paracentrotus lividus

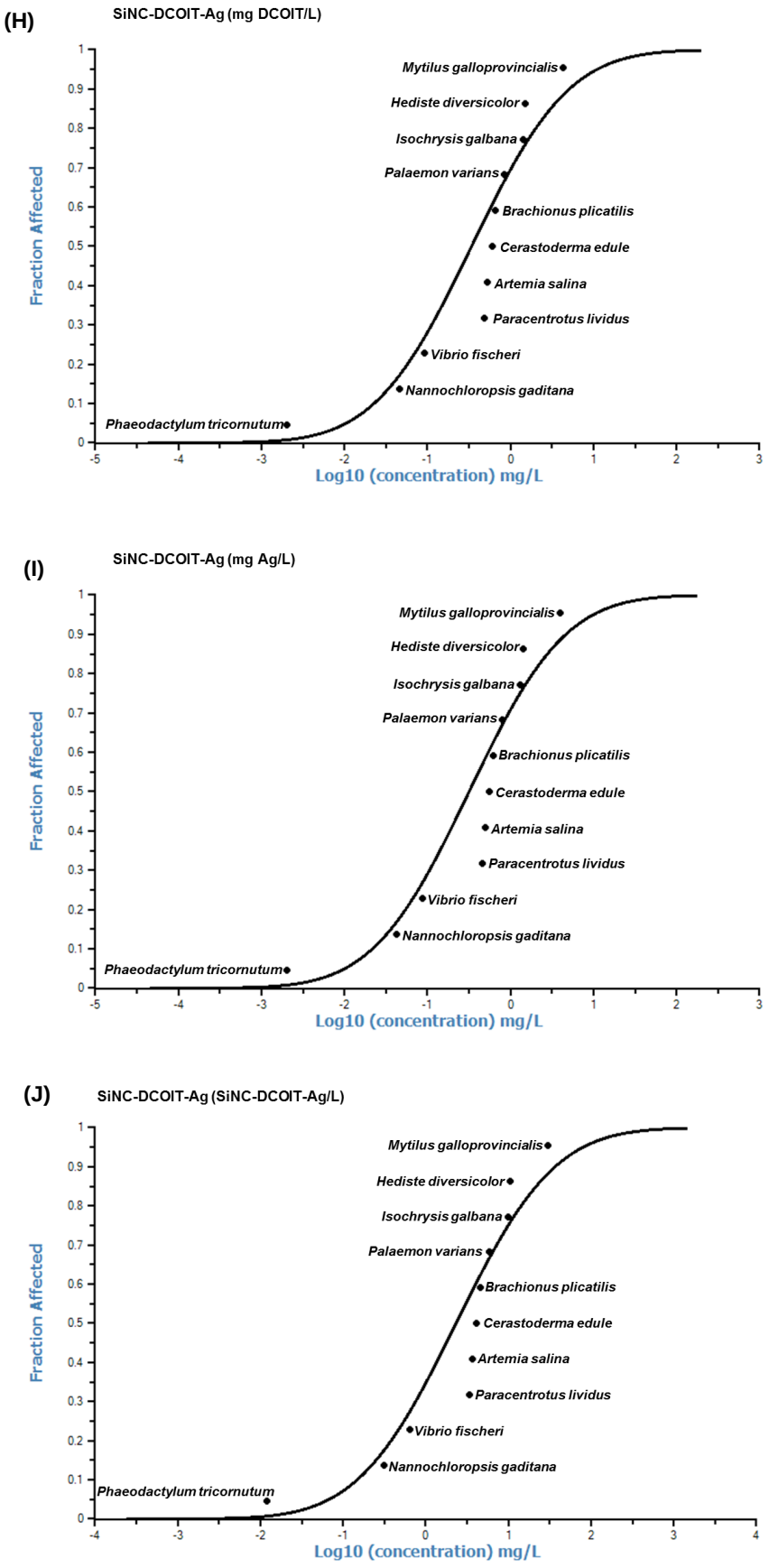


Figure 3.1 – Species sensitivity distributions for: (A) SiNC; (B) DCOIT; (C) Ag^{+}; (D) SiNC-DCOIT (as DCOIT content); (E) SiNC-DCOIT (as heterogenous); (F) SiNC-Ag (as Ag^{+} content); (G) SiNC-Ag (as heterogenous); (H) SiNC-DCOIT-Ag (as DCOIT content); (I) SiNC-DCOIT-Ag (as Ag^{+} content); (J) SiNC-DCOIT-Ag (as heterogenous) using L/E/IC_{50} data for different species.

The SSDs constructed with L/E/IC_{50} data are shown in Figure 3.1 (A to J) and the respective estimated HC_5 values in Table 3.2. The lowest HC_5 value was observed for the free DCOIT (0.001 mg DCOIT/L) and the highest was for SiNC-Ag (0.187 mg SiNC-Ag/L). Moreover, comparing the encapsulated forms of DCOIT and Ag^+ the value of HC_5 is higher when they are encapsulated alone (23-fold and 2.7-fold higher in the forms SiNC-DCOIT and SiNC-Ag, respectively). Regarding SiNC-DCOIT-Ag, a HC_5 value 9-fold higher than for the free form was calculated for DCOIT but for silver the HC_5 of the encapsulated compound is the same as the free Ag^+. It is further noted that for the silica capsules loaded with the two biocides (SiNC-DCOIT-Ag) the HC_5 value (of the corresponding biocide) is approximately 2.6 times lower compared to both SiNC-DCOIT or SiNC-Ag. As heterocompounds, SiNC-Ag was the one presenting the higher HC_5, followed by SiNC-DCOIT and SiNC-DCOIT-Ag.

Regarding SiNC, the analysis demonstrated that the bryozoan *Bugula neritina* was the most sensitive species whereas the crustacean *Palaemon varians* was the less sensitive. The polychaete *Hediste diversicolor* is the most tolerant species to DCOIT and silver, while the microalgae *Emiliana huxleyi* and dinoflagellate *Lingulodinium polyedrum* were the most sensitive species, respectively. Regarding the two encapsulated forms of DCOIT (SiNC-DCOIT and SiNC-DCOIT-Ag) the diatom *Phaeodactylum tricornutum* was the most sensitive species and the bivalve *Mytilus galloprovincialis* was the less sensitive in both cases. This also applies to the encapsulated form of silver SiNC-DCOIT-Ag. In case of SiNC-Ag, the crustacean *Artemia salina* was the less sensitive, whereas the sea-urchin *Paracentrotus lividus* was the most sensitive.

The highest calculated PNEC values were recorded for SiNC-Ag ($PNEC_{stat}$ = 0.037 and $PNEC_{determ}$ = 0.0001 mg SiNC-Ag/L) and the lowest for DCOIT ($PNEC_{stat}$ = 0.0002 and $PNEC_{determ}$ = 0.0000004 mg DCOIT/L).

Table 3.2 – Hazardous concentrations 5% (HC_5) obtained from species sensitivity distributions using L/E/IC_{50} data for the different species and PNEC values. n is the sample size and tests for normality included: the Anderson–Darling test, the Kolmogorov–Smirnov test and the Cramer von Mises test. $PNEC_{stat}$ – derived from the HC_5 with AF=5; $PNEC_{determ}$ – derived from the lowest L/E/IC_{50} with AF=1000 (according to TGD (2003)).

Contaminant	Units	n	HC_5	CI 95%	Normality tests	$PNEC_{stat}$	$PNEC_{determ}$
SiNC	mg SiNC/L	13[a]	0.101	0.012 – 0.378	Accepted[b]	0.020	0.000001
DCOIT	mg DCOIT/L	34[c]	0.001	0.0003 – 0.019	Accepted[d]	0.0002	0.0000004
Ag	mg Ag^+/L	46[c]	0.009	0.005 – 0.015	Accepted	0.002	0.000006
SiNC-DCOIT	mg DCOIT/L	11[a]	0.023	0.002 – 0.100	Accepted	0.005	0.000007
	mg SiNC/L	11[a]	0.100	0.008 – 0.437	Accepted	0.020	0.000029
	mg SiNC-DCOIT/L	11[a]	0.123	0.010 – 0.538	Accepted	0.025	0.000036
SiNC-Ag	mg Ag^+/L	11[a]	0.024	0.004 – 0.069	Accepted[e]	0.005	0.000017
	mg SiNC/L	11[a]	0.144	0.023 – 0.419	Accepted[e]	0.029	0.000109
	mg SiNC-Ag/L	11[a]	0.187	0.030 – 0.543	Accepted[e]	0.037	0.0001
SiNC-DCOIT-Ag	mg DCOIT/L	11[a]	0.009	0.001 – 0.038	Accepted[f]	0.002	0.000002
	mg Ag^+/L	11[a]	0.009	0.001 – 0.036	Accepted[f]	0.002	0.000002
	mg SiNC/L	11[a]	0.044	0.004 – 0.173	Accepted[f]	0.009	0.00001
	mg SiNC-DCOIT-Ag/L	11[a]	0.061	0.005 – 0.248	Accepted[f]	0.012	0.00001

[a] Only one EC_{50} value available for each species.

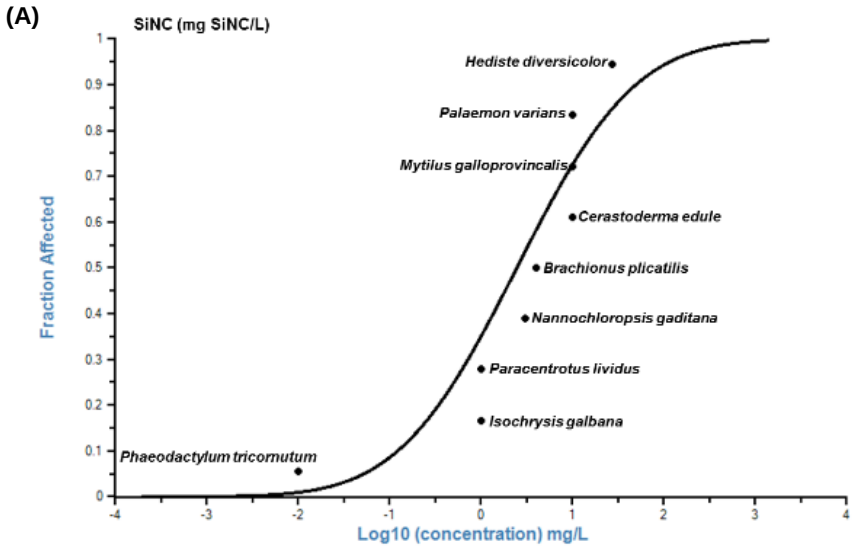
[b] Except for Anderson-Darling test for normality where significance level was 0.01 and Cramer von Mises test that rejected 0.1 significance level.

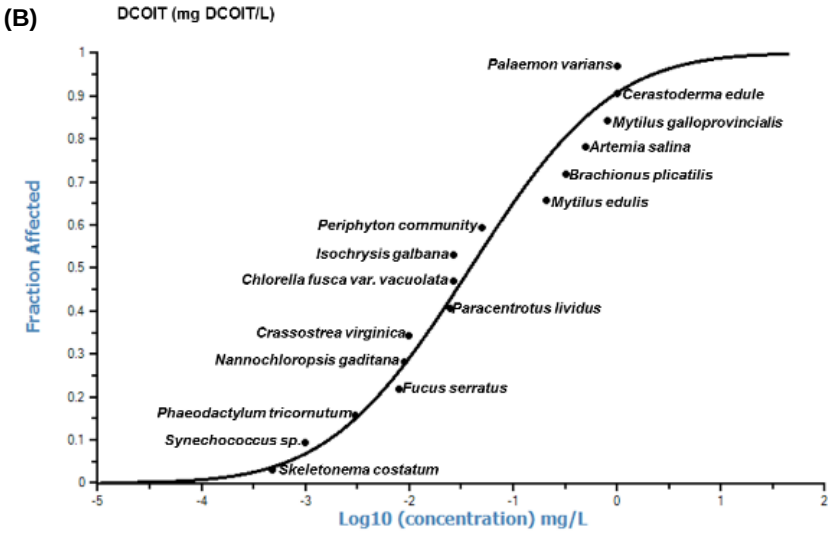
[c] Only one EC50 value available for several species, thus the calculation of the geometric mean was not possible in these cases.

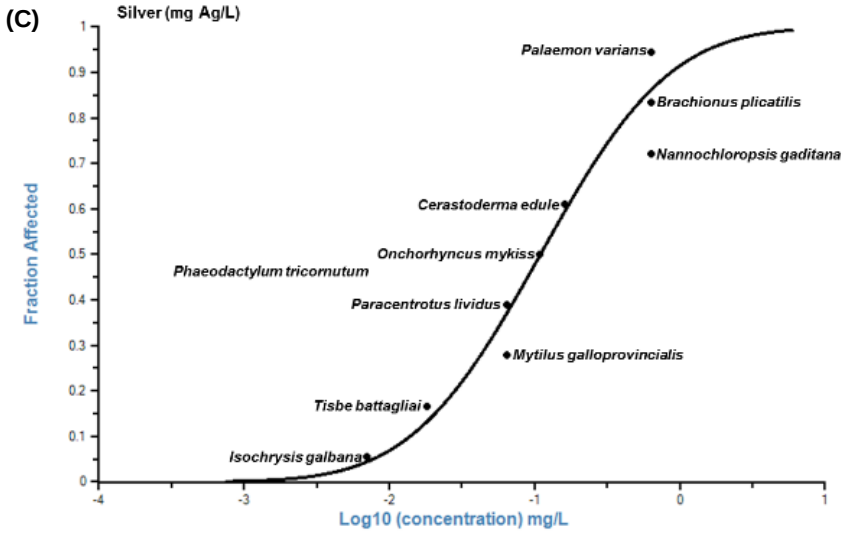
[d] Except for Kolmogorov–Smirnov test where significance level was 0.025 and 0.01 and for Anderson–Darling test that rejected 0.1 significance level.

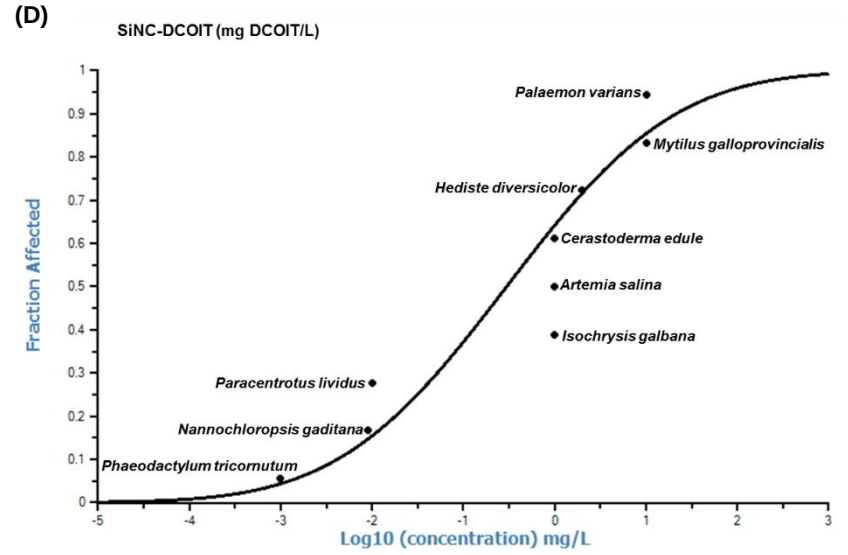
[e] Except for significance level 0.1 in Anderson-Darling, Kolmogorov–Smirnov and Cramer von Mises tests.

[f] Except for Kolmogorov–Smirnov test for normality that rejected all significance levels and for Anderson–Darling and Cramer von Mises tests where significance level was 0.025 and 0.01

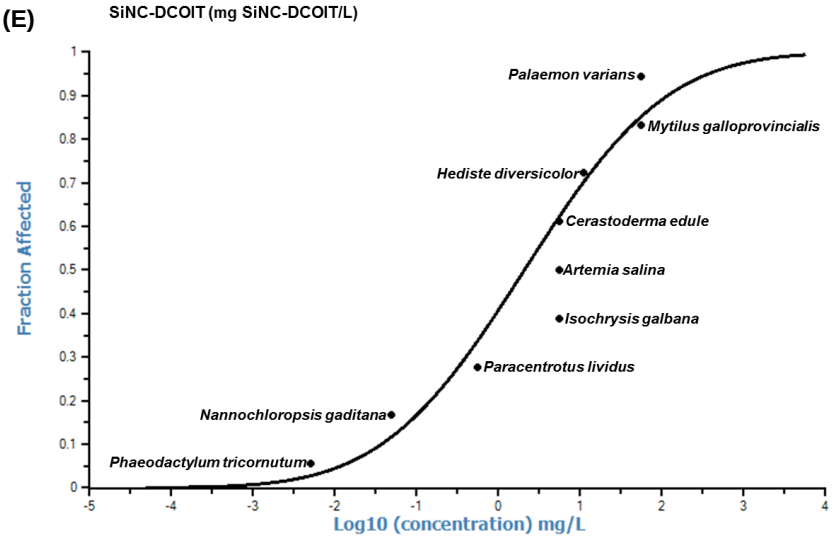



(B)
DCOIT (mg DCOIT/L)
Fraction Affected
Log10 (concentration) mg/L
Palaemon varians
Cerastoderma edule
Mytilus galloprovincialis
Artemia salina
Brachionus plicatilis
Mytilus edulis
Periphyton community
Isochrysis galbana
Chlorella fusca var. vacuolata
Paracentrotus lividus
Crassostrea virginica
Nannochloropsis gaditana
Fucus serratus
Phaeodactylum tricornutum
Synechococcus sp.
Skeletonema costatum

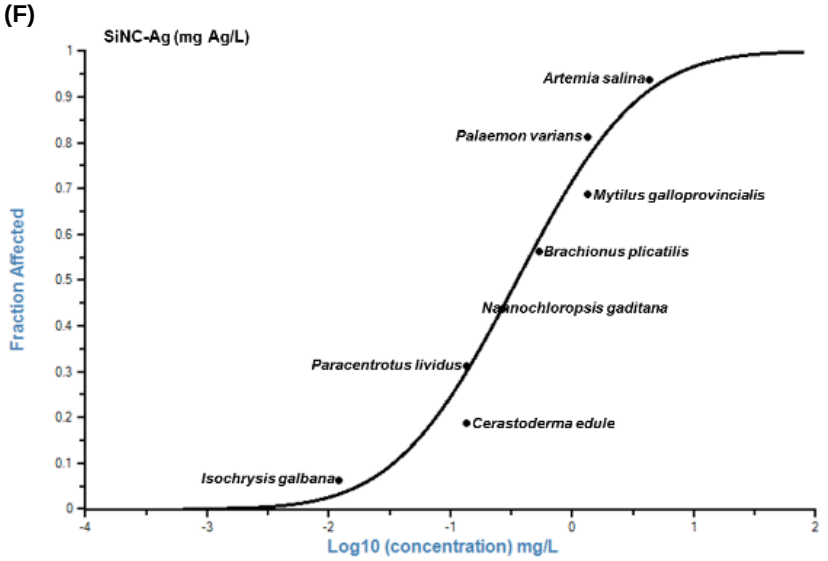

(C)
Silver (mg Ag/L)
Fraction Affected
Log10 (concentration) mg/L
Palaemon varians
Brachionus plicatilis
Nannochloropsis gaditana
Cerastoderma edule
Onchorhyncus mykiss
Phaeodactylum tricornutum
Paracentrotus lividus
Mytilus galloprovincialis
Tisbe battagliai
Isochrysis galbana


(D)
SiNC-DCOIT (mg DCOIT/L)
Fraction Affected
Log10 (concentration) mg/L
Palaemon varians
Mytilus galloprovincialis
Hediste diversicolor
Cerastoderma edule
Artemia salina
Isochrysis galbana
Paracentrotus lividus
Nannochloropsis gaditana
Phaeodactylum tricornutum

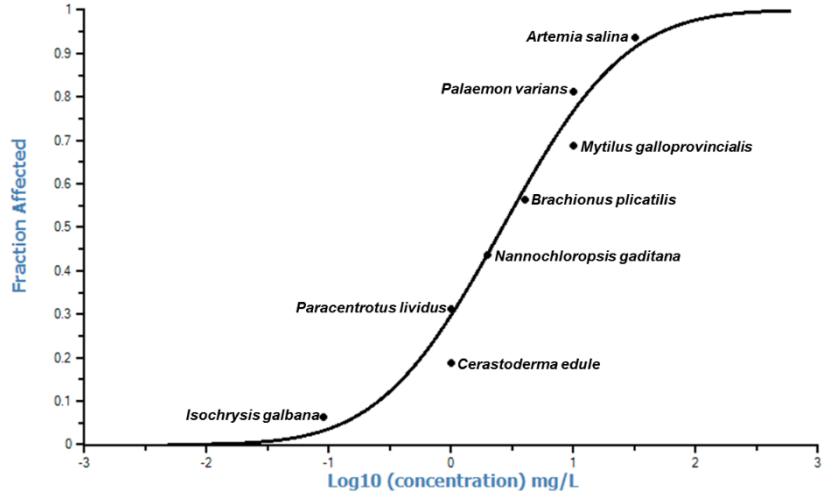
(E)


SiNC-DCOIT (mg SiNC-DCOIT/L)
Fraction Affected
Log10 (concentration) mg/L
Palaemon varians
Mytilus galloprovincialis
Hediste diversicolor
Cerastoderma edule
Artemia salina
Isochrysis galbana
Paracentrotus lividus
Nannochloropsis gaditana
Phaeodactylum tricornutum

(F)


SiNC-Ag (mg Ag/L)
Fraction Affected
Log10 (concentration) mg/L
Artemia salina
Palaemon varians
Mytilus galloprovincialis
Brachionus plicatilis
Nannochloropsis gaditana
Paracentrotus lividus
Cerastoderma edule
Isochrysis galbana

(G)


SiNC-Ag (mg SiNC-Ag/L)
Fraction Affected
Log10 (concentration) mg/L
Artemia salina
Palaemon varians
Mytilus galloprovincialis
Brachionus plicatilis
Nannochloropsis gaditana
Paracentrotus lividus
Cerastoderma edule
Isochrysis galbana

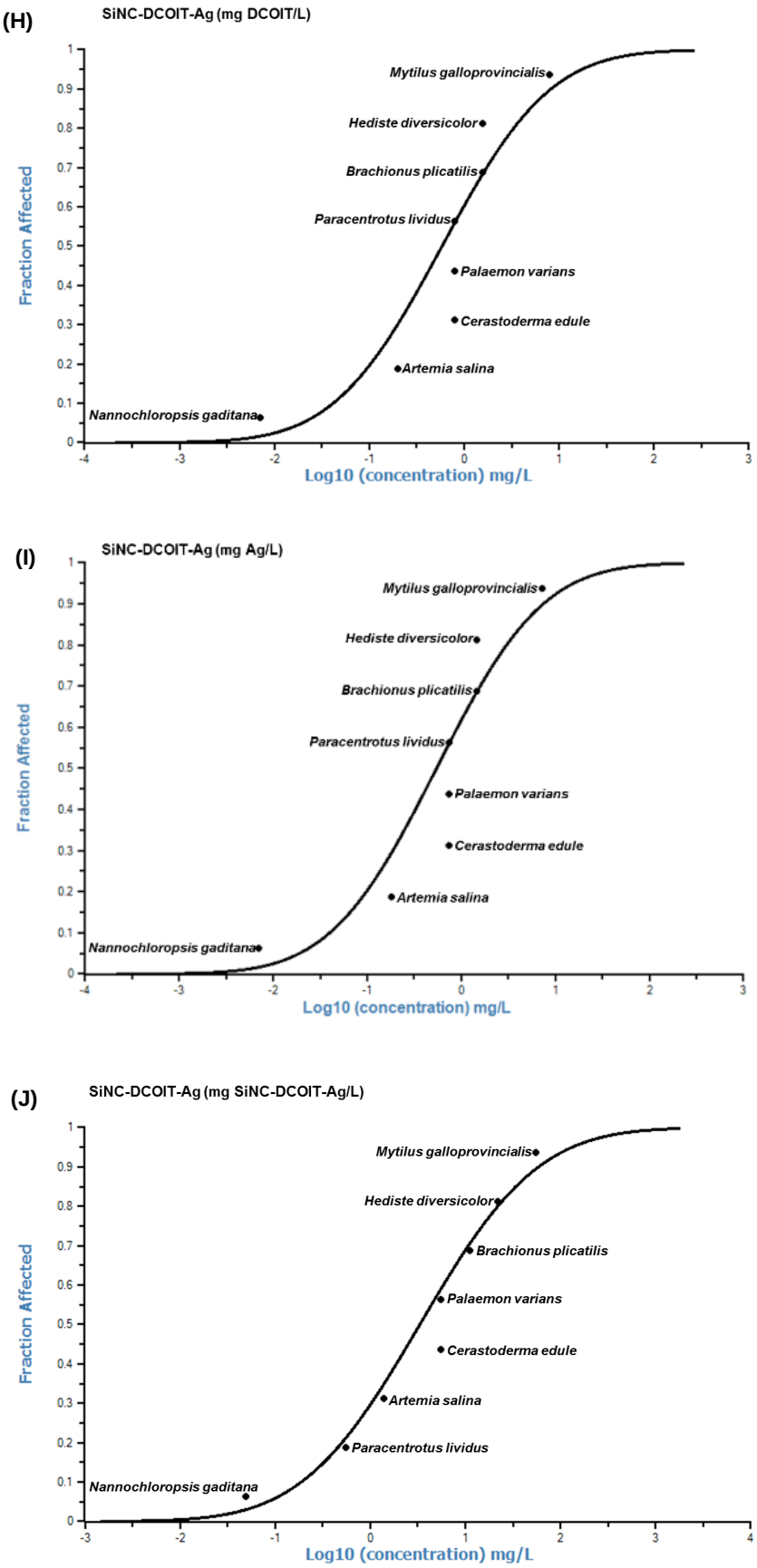


Figure 3.2 – Species sensitivity distributions for: (A) SiNC; (B) DCOIT; (C) Ag^{+}; (D) SiNC-DCOIT (as DCOIT content); (E) SiNC-DCOIT (as heterogenous); (F) SiNC-Ag (as Ag^{+} content); (G) SiNC-Ag (as heterogenous); (H) SiNC-DCOIT-Ag (as DCOIT content); (I) SiNC-DCOIT-Ag (as Ag^{+} content); (J) SiNC-DCOIT-Ag (as heterogenous) using NOEC data for different species.

Regarding the SSDs constructed with NOEC values, the obtained graphs are shown in Figure 3.2 (A to J) and the estimated HC_5 values in Table 3.3. As for those constructed with L/E/IC_{50}, the lowest HC_5 value was observed for free DCOIT (0.001 mg DCOIT/L) and the highest was for SiNC-Ag (0.114 mg SiNC-Ag/L).

Comparing the encapsulated forms of DCOIT with the free biocide, for SiNC-DCOIT an HC_5 value equal to the free form was estimated, whereas for SiNC-DCOIT-Ag, the HC_5 value was 10-fold higher comparing with the free form. Regarding silver, the HC_5 value was higher for both encapsulated forms comparing to free Ag^+. The increase was of 2-fold for SiNC-Ag and 1.4-fold for SiNC-DCOIT-Ag. On the other hand, unlike in the curves constructed with L/E/IC_{50} data, the encapsulated forms SiNC-DCOIT and SiNC-Ag showed a lower value of HC_5 (of the corresponding biocide) than SiNC-DCOIT-Ag. This value is especially different in case of SiNC-DCOIT, as it is 10 times lower than for SiNC-DCOIT-Ag. As heterocompounds, SiNC-Ag was the one presenting the higher HC_5, followed by SiNC-DCOIT-Ag and SiNC-DCOIT.

Regarding SiNC, the analysis demonstrated that the microalgae *Phaeodactylum tricornutum* was the most sensitive species whereas the polychaete *Hediste diversicolor* was the less sensitive. The graphs constructed for DCOIT and silver show that the microalgae *Skeletonema costatum* and *Isochrysis galbana* were the most susceptible species, respectively, while the crustacean *Palaemon varians* was the most tolerant to both biocides. In case of the encapsulated forms of these biocides, microalgae species *Phaodactylum tricornutum*, *Isochrysis galbana* and *Nannochloropsis gaditana* were the most sensitive to SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag, respectively, whereas *Palaemon varians*, *Artemia salina* and *Mytilus galloprovincialis* were the less sensitive.

The highest calculated PNEC values were recorded for SiNC-Ag ($PNEC_{stat}$ = 0.023 mg and $PNEC_{determ}$ = 0.001 SiNC/L) and the lowest for DCOIT ($PNEC_{stat}$ = 0.0002 mg and $PNEC_{determ}$ = 0.0000048 DCOIT/L).

Table 3.3 – Hazardous concentrations 5% (HC_5) obtained from species sensitivity distributions using NOEC data for the different species and PNEC values. n is the sample size and tests for normality included: the Anderson–Darling test, the Kolmogorov–Smirnov test and the Cramer von Mises test. $PNEC_{stat}$ – derived from the HC_5 with AF=5; $PNEC_{determ}$ – derived from the lowest NOEC with AF=100 (according to TGD (2003)).

Contaminant	Units	n	HC_5	CI 95%	Normality tests	$PNEC_{stat}$	$PNEC_{determ}$
SiNC	mg SiNC/L	9[a]	0.044	0.002 – 0.242	Accepted[b]	0.009	0.0001
DCOIT	mg DCOIT/L	15[c]	0.001	0.0001 – 0.002	Accepted	0.0002	0.0000048
Ag	mg Ag^+/L	9[c]	0.007	0.001 – 0.022	Accepted	0.001	0.00007
SiNC-DCOIT	mg DCOIT/L	9[a]	0.001	0.00001 – 0.011	Accepted[d]	0.0002	0.00001
	mg SiNC/L	9[a]	0.008	0.0001 – 0.075	Accepted[d]	0.002	0.00004
	mg SiNC-DCOIT	9[a]	0.009	0.0002 – 0.092	Accepted[d]	0.002	0.0001
SiNC-Ag	mg Ag^+/L	8[a]	0.015	0.001 – 0.061	Accepted	0.003	0.00012
	mg SiNC/L	8[a]	0.089	0.006 – 0.359	Accepted	0.018	0.0007
	mg SiNC-Ag	8[a]	0.114	0.008 – 0.458	Accepted	0.023	0.001
SiNC-DCOIT-Ag	mg DCOIT/L	8[a]	0.010	0.0004 – 0.056	Accepted	0.002	0.00007
	mg Ag^+/L	8[a]	0.010	0.0004 – 0.052	Accepted	0.002	0.00007
	mg SiNC/L	8[a]	0.045	0.002 – 0.250	Accepted	0.009	0.00032
	mg SiNC-DCOIT-Ag	8[a]	0.071	0.003 – 0.388	Accepted	0.014	0.0005

[a] Only one EC_{50} value available for each species

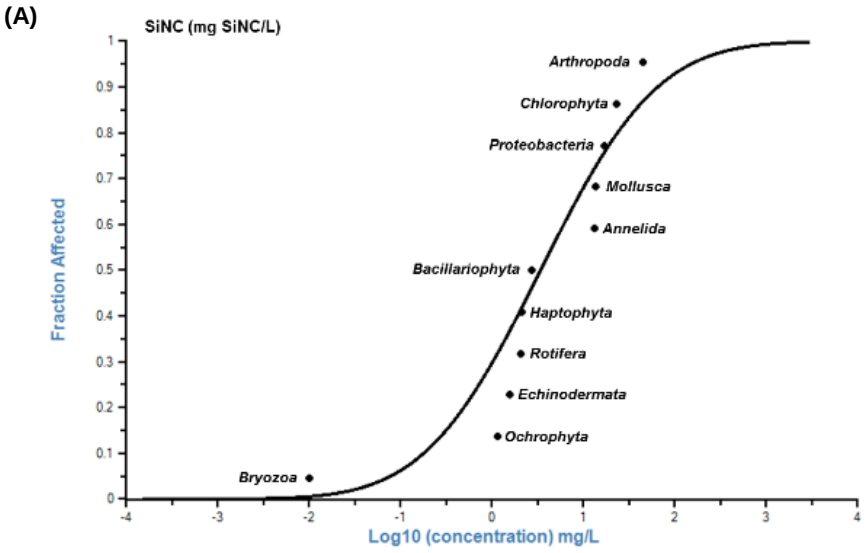
[b] Except for Anderson-Darling test for normality where significance level was 0.025 and 0.01 and Cramer von Mises test that rejected 0.1 significance level.

[c] With the exception of *Mytilus galloprovincialis* and *Paracentrotus lividus* for DCOIT and *Tisbe batagliai* for Ag^+, only one EC_{50} value available for each species.

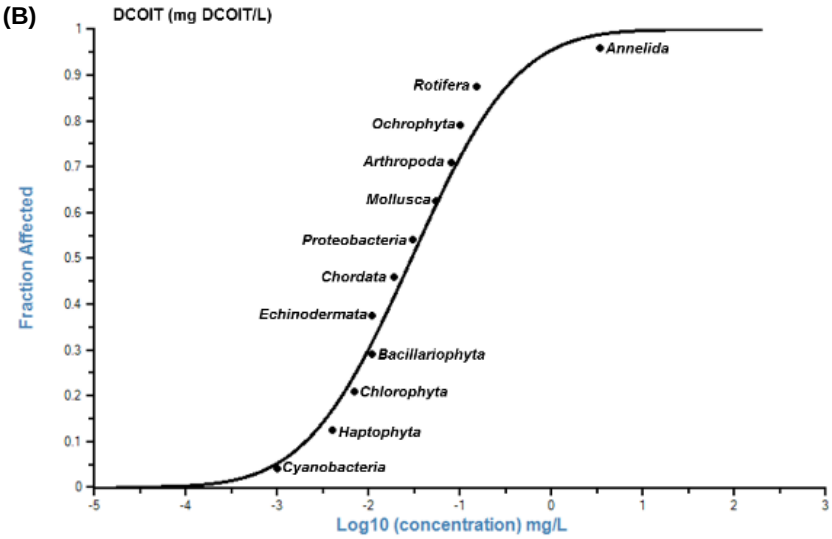
[d] Except for Anderson-Darling and Cramer von Mises tests that rejected 0.01 significance level and for Kolmogorov–Smirnov test that only accepted 0.01 significance level.

[e] Except for Kolmogorov–Smirnov test where significance level was 0.025 and 0.01 and for Anderson–Darling and Cramer von Mises tests where that rejected 0.1 significance level.

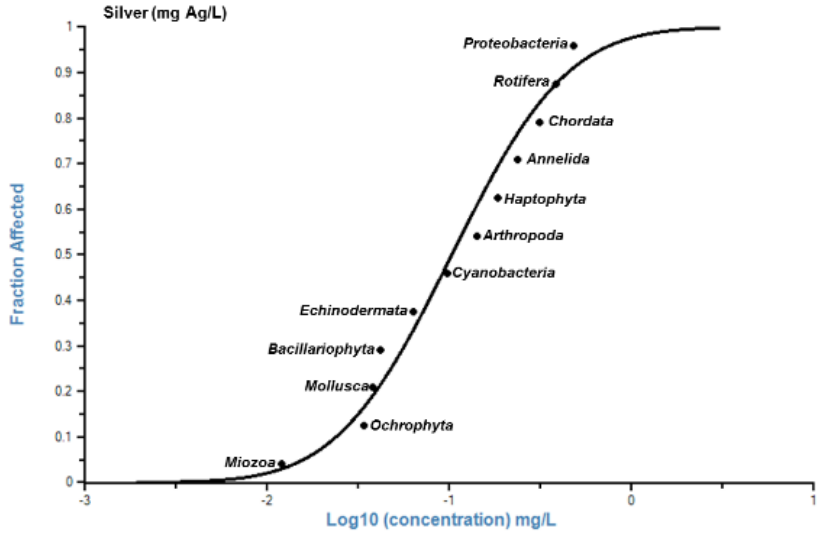
3.4.2. Species sensitivity distributions (SSDs) – data grouped by phylum



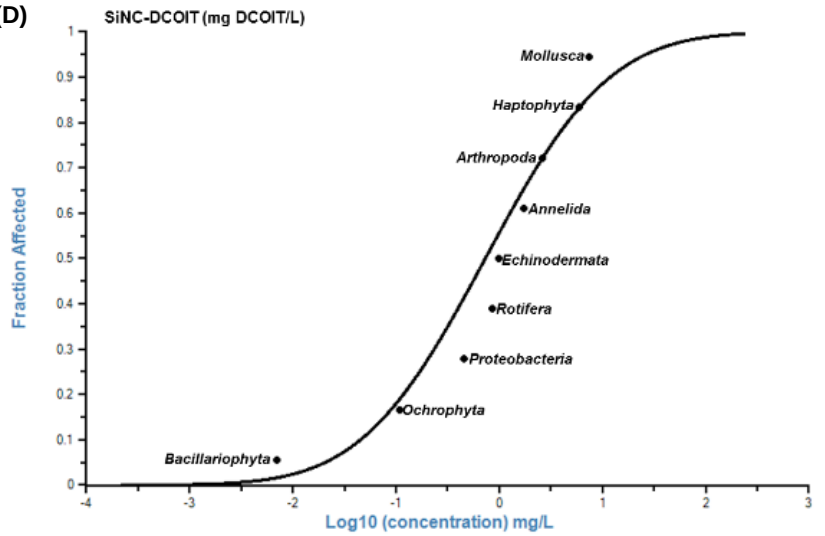
(B)


DCOIT (mg DCOIT/L)
Annelida
Rotifera
Ochrophyta
Arthropoda
Mollusca
Proteobacteria
Chordata
Echinodermata
Bacillariophyta
Chlorophyta
Haptophyta
Cyanobacteria
Fraction Affected
Log10 (concentration) mg/L

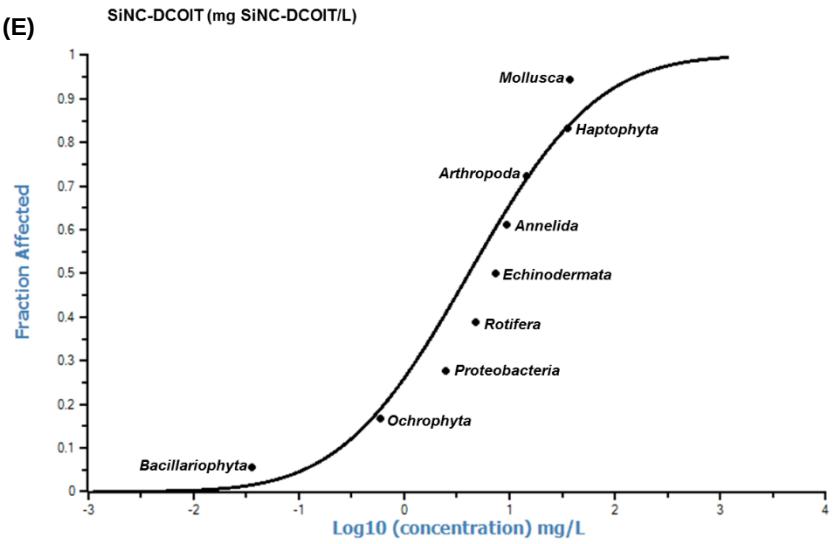
(C)


Silver (mg Ag/L)
Proteobacteria
Rotifera
Chordata
Annelida
Haptophyta
Arthropoda
Cyanobacteria
Echinodermata
Bacillariophyta
Mollusca
Ochrophyta
Miozoa
Fraction Affected
Log10 (concentration) mg/L

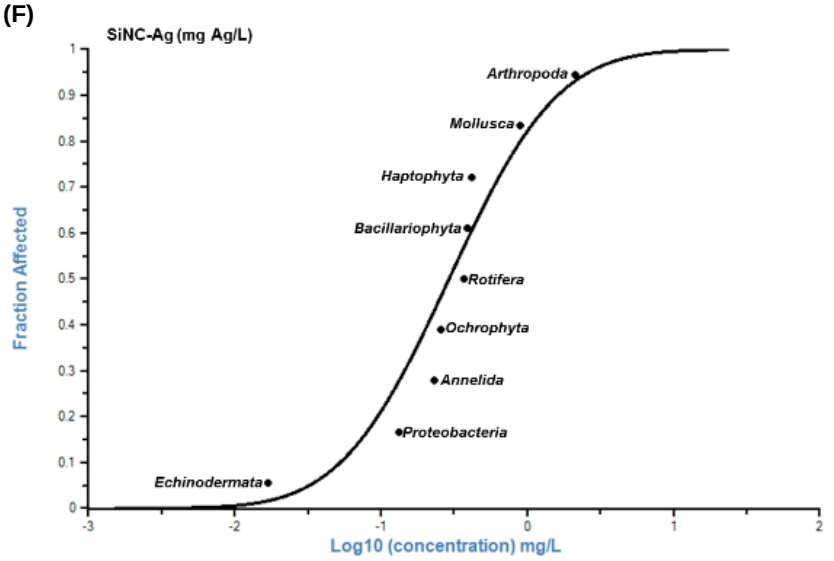
(D)


SiNC-DCOIT (mg DCOIT/L)
Mollusca
Haptophyta
Arthropoda
Annelida
Echinodermata
Rotifera
Proteobacteria
Ochrophyta
Bacillariophyta
Fraction Affected
Log10 (concentration) mg/L

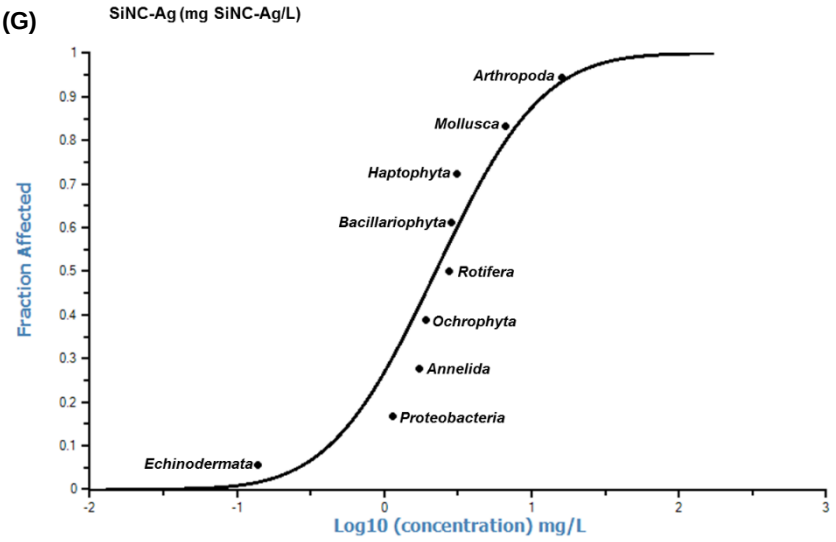
(E)


SiNC-DCOIT (mg SiNC-DCOIT/L)
Fraction Affected
Log10 (concentration) mg/L
Mollusca
Haptophyta
Arthropoda
Annelida
Echinodermata
Rotifera
Proteobacteria
Ochrophyta
Bacillariophyta

(F)


SiNC-Ag (mg Ag/L)
Fraction Affected
Log10 (concentration) mg/L
Arthropoda
Mollusca
Haptophyta
Bacillariophyta
Rotifera
Ochrophyta
Annelida
Proteobacteria
Echinodermata

(G)


SiNC-Ag (mg SiNC-Ag/L)
Fraction Affected
Log10 (concentration) mg/L
Arthropoda
Mollusca
Haptophyta
Bacillariophyta
Rotifera
Ochrophyta
Annelida
Proteobacteria
Echinodermata

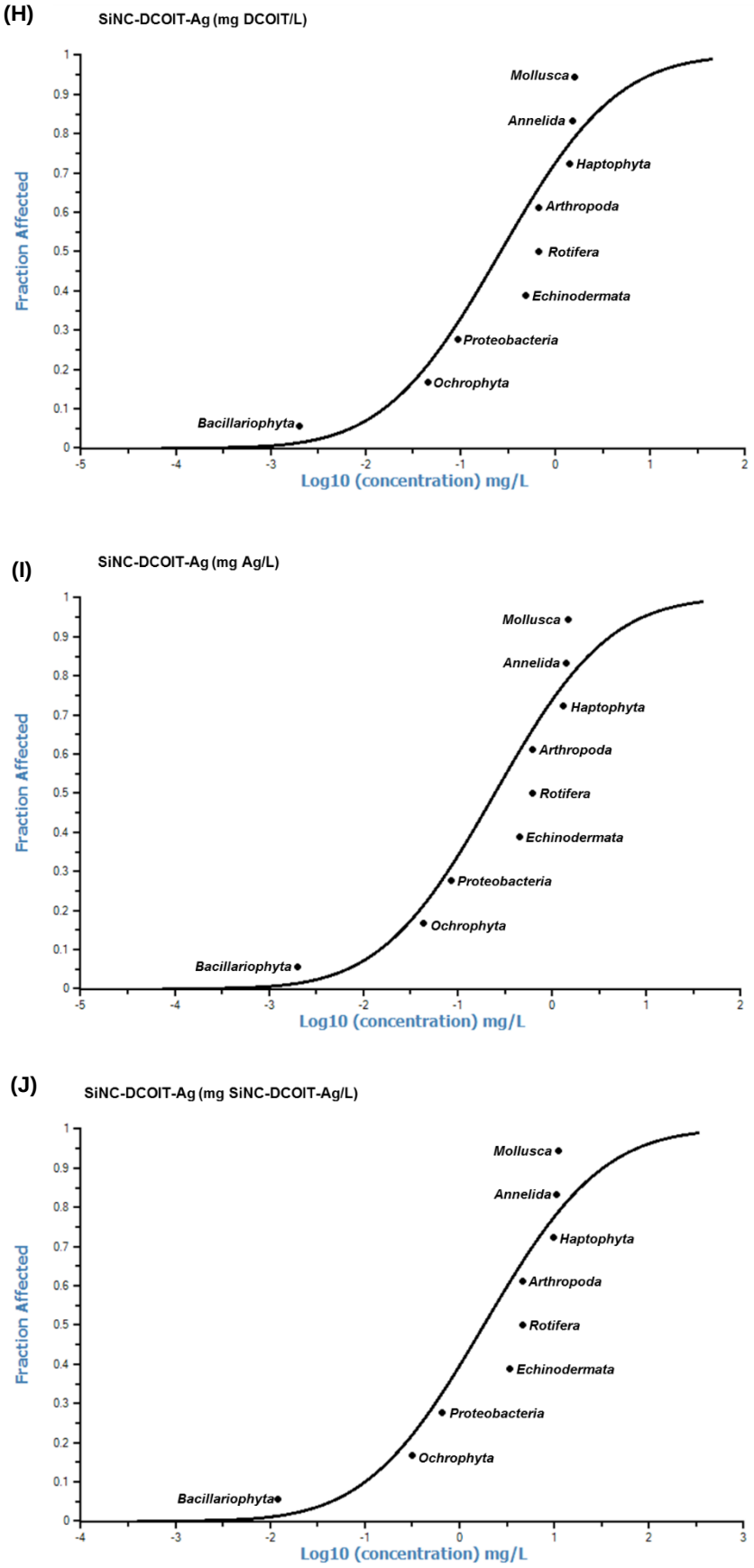


Figure 3.3 – Species sensitivity distributions for: (A) SiNC; (B) DCOIT; (C) Ag^{+}; (D) SiNC-DCOIT (as DCOIT content); (E) SiNC-DCOIT (as heterogenous); (F) SiNC-Ag (as Ag^{+} content); (G) SiNC-Ag (as heterogenous); (H) SiNC-DCOIT-Ag (as DCOIT content); (I) SiNC-DCOIT-Ag (as Ag^{+} content); (J) SiNC-DCOIT-Ag (as heterogenous) using L/E/I/C_{50} data for different phylum.

Figure 3.3 (A to J) show the SSD graphs obtained with L/E/I/C_{50} data grouped by phylum and the estimated HC_5 values are present in Table 3.4. The lowest HC_5 value was observed for free DCOIT (0.001 mg DCOIT/L) and the highest was for SiNC-Ag (0.241 mg SiNC-Ag/L). In case of DCOIT, the encapsulated forms SiNC-DCOIT and SiNC-DCOIT-Ag, showed a HC_5 value 18 and 6 times higher than the free form, respectively. Regarding silver, it was only observed an increase of 2 times in this value for SiNC-Ag. In case of SiNC-DCOIT-Ag, free silver showed a 2.5-fold higher HC_5 comparing with the encapsulated form. Comparing the silica nanocapsules loaded with the two biocides with the nanomaterial loaded only with one active compound, SiNC-DCOIT-Ag showed 3 and 5 times lower HC_5 values, of the respective biocide, comparing with SiNC-DCOIT and SiNC-Ag, respectively. As heterocompounds, SiNC-Ag was the one presenting the higher HC_5, followed by SiNC-DCOIT and SiNC-DCOIT-Ag.

Bryozoa was the most sensitive phylum to SiNC, while Arthropoda was the more tolerant. Regarding the unloaded biocides, Cyanobacteria and Miozoa were the most sensitive to DCOIT and silver whereas Annelida and Proteobacteria were the less sensitive, respectively. In case of the encapsulated forms of the biocides, Bacillariophyta and Mollusca were the more and less susceptible to SiNC-DCOIT and SiNC-DCOIT-Ag, while Echinodermata and Arthropoda where the more and less sensitive phyla to SiNC-Ag.

The highest calculated PNEC values were recorded for SiNC-Ag ($PNEC_{stat}$ = 0.048 and $PNEC_{determ}$ = 0.0001 mg SiNC-Ag/L) and the lowest for DCOIT ($PNEC_{stat}$ = 0.0002 and $PNEC_{determ}$ = 0.000004 mg DCOIT/L).

Table 3.4 – Hazardous concentrations 5% (HC_5) obtained from species sensitivity distributions using $L/E/IC_{50}$ data for different phylum and PNEC values. n is the sample size and tests for normality included: the Anderson–Darling test, the Kolmogorov–Smirnov test and the Cramer von Mises test. PNECstat – derived from the HC5 with AF=5; $PNEC_{determ}$ – derived from the lowest $L/E/IC_{50}$ with AF=1000 (according to TGD (2003)).

Contaminant	Units	n	HC_5	CI 95%	Normality tests	$PNEC_{stat}$	$PNEC_{determ}$
SiNC	mg SiNC/L	11[a]	0.069	0.005 – 0.309	Accepted[b]	0.014	0.000001
DCOIT	mg DCOIT/L	12[a]	0.001	0.0001 – 0.003	Accepted	0.0002	0.0000004
Ag	mg Ag^+/L	12[a]	0.015	0.005 – 0.031	Accepted	0.003	0.000006
SiNC-DCOIT	mg DCOIT/L	9[a]	0.018	0.001 – 0.087	Accepted	0.004	0.000007
	mg SiNC/L	9[a]	0.078	0.004 – 0.379	Accepted	0.016	0.000029
	mg SiNC-DCOIT	9[a]	0.096	0.005 – 0.467	Accepted	0.019	0.000036
SiNC-Ag	mg Ag^+/L	9[a]	0.030	0.005 – 0.078	Accepted	0.006	0.000017
	mg SiNC/L	9[a]	0.184	0.032 – 0.473	Accepted	0.037	0.000109
	mg SiNC-Ag	9[a]	0.241	0.043 – 0.615	Accepted	0.048	0.0001
SiNC-DCOIT-Ag	mg DCOIT/L	9[a]	0.006	0.0003 – 0.030	Accepted[c]	0.001	0.000002
	mg Ag^+/L	9[a]	0.006	0.0003 – 0.028	Accepted[c]	0.001	0.000002
	mg SiNC/L	9[a]	0.028	0.002 – 0.137	Accepted[c]	0.006	0.00001
	mg SiNC-DCOIT-Ag	9[a]	0.038	0.002 – 0.194	Accepted[c]	0.008	0.00001

[a] Only one $L/E/IC_{50}$ value available for several phyla.

[b] Except for Anderson-Darling test for normality where significance level was 0.025 and 0.01.

[c] Except for Anderson-Darling and Kolmogorov–Smirnov tests where significance level was 0.025 and 0.01 and for Cramer von Mises test that rejected 0.1 significance level.

Please note that no NOEC based SSDs were calculated due to the number of phyla being lower than the recommended in the literature (8-10).

3.5. Discussion

After the extensive data search for the construction of the SSDs, no data were found for *I. galbana*, *N. gaditana*, *P. tricornutum*, *B. plicatilis*, *C. edule*, *H. diversicolor*, *A. salina* and *P. varians* for DCOIT, as well for silver (excepting for *I. galbana* and *H. diversicolor*). No data was also found for *P. lividus* for silver. These species are ecological relevant in marine ecosystems and play different important roles in the function and structure of ecosystems, thus this study is important to improve the quality and amount of data related to the toxicity of these compounds and to improve the realism of distribution-based hazard and risk assessments.

Moreover, besides this study, only one SSD with marine organisms have been constructed for DCOIT. Mochida et al. (2015) calculated a HC_5 value (based on acute $L/E/IC_{50}$ data) of 0.0005 mg DCOIT/L, which is two times lower than the obtained in this study (Table 3.2S). However, the methodology used by Mochida et al. (2015) to construct the SSD was different than the used in the present study. On the other hand, in this study, additional data (for 6 more species) were used to construct the curve, which can influence the HC_5 value and it is widely known that the greater the amount the more reliable are the obtained estimates. Comparing with other biocides, Mochida et al. (2012) estimated a HC_5 value for pyridine triphenylborane (PTPB) lower than the estimated value for DCOIT (Table 3.2S), showing that using this approach DCOIT appears to be slightly less hazardous than PTPB. A HC_5 value (based on chronic LOEC and NOEC from a marine dataset) for TBTO was estimated as 250 times lower than the estimated HC_5 value for DCOIT (based on acute and short-term chronic NOEC data) (Leung et al., 2007). This expected lower toxicity and prediction of risk led to the replacement of TBT by DCOIT.

Regarding silver, a value of HC_5 18 times lower than the obtained in this study (with $L/E/IC_{50}$ data) was found in literature for marine species (Table 3.2S). This difference may be due to the difference in the amount of data used to construct the SSD in the two studies; the present study included data for 28 additional species comparing with CCME (2005). CCME (2005) also estimated a SSD for freshwater species and obtained a HC_5 value 1.13 times lower, suggesting that those species are more susceptible to silver than marine species.

Comparing the obtained deterministic PNEC values with values collected from literature, the estimated PNEC for DCOIT in this study is equal to the estimated by Mochida et al. (2015). On the other hand, contrary to what was observed for the HC_5 value, the estimated PNEC value for pyridine triphenylborane (PTPB) was 75 times higher than the estimated value for DCOIT (Table 3.2S). This shows that between these two booster biocides, DCOIT appears to be more hazardous.

On the other hand, for the curves made with $L/E/IC_{50}$ data, it was expected that target species (namely *V. fischeri*, *Skeletonema costatum*, *M. galloprovincialis*, *M. edulis* and *A. amphitrite*) and silver (namely *V. fischeri* and *Synechococcus* sp.) would be more sensitive to these biocides and positioned lower on the curve relatively to non-target species. This result shows that silver appears to have a broader spectrum of action against marine species besides its bactericidal activity and that it seems to be even more efficient against non-microbial species, representing a potential hazard to marine ecosystems. Regarding DCOIT, this result confirms its well-known broad-spectrum action posing some concern to the ecosystem since it seems to affect non-target

organisms even at lower concentrations than fouler species, namely organisms that are at the base of the trophic chain (such as microalgae, copepods, rotifers and macroalgae) and higher non-target organisms (such as echinoderms and fish). This impairment at both lower and higher levels of the trophic chains can represent a potential disruption in the functioning of the ecosystem. Possibly, this happens due to the mode of action of DCOIT as, besides interfering with other metabolic pathways, it inhibits glutathione reductase by irreversible binding to the enzyme active loci (Williams, 2007; Hellio and Yebra, 2009; Wendt et al., 2016).

The PNEC results obtained for both biocides demonstrate the need to develop novel high performance and eco-friendly solutions for their incorporation in coatings. For the encapsulated forms of DCOIT and silver, with the exception of *M. galloprovincialis* which was amongst the less susceptible species, it was found that the target species *V. fischeri* and *P. tricornutum* are among the most sensitive to SiNC-DCOIT and SiNC-DCOIT-Ag, as well as the bacteria species to SiNC-Ag. As mentioned before, the results obtained for the mussel species and the low sensititivy that these organisms present to some chemical compounds is usually related to their biological mechanism of closing the valves to avoid contaminants' exposure.

In general, these are promising results, as encapsulation worked to increase efficacy against fouler species, while decreasing the toxicity of biocides to non-target species. However, curves derived from NOEC values did not allow to corroborate these findings due to the impossibility of calculating NOEC values for some target and non-target species, so this result should be interpreted with caution.

Moreover, in the approaches used to generate the SSDs (L/E/IC_{50} or NOEC data grouped by species or by phylum), SiNC-Ag presented the highest HC_5 value (as heterocompound) and free DCOIT the lowest value, followed by free silver. The HC_5 value for the encapsulated forms of DCOIT and silver (SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag) was higher comparing with their free forms (excepting for Ag^+ in the form SiNC-DCOIT-Ag that presented an equal value, for SSD constructed for different species). This result indicates that the encapsulation seems to be a promising technique to reduce ecological hazard of these biocides.

Comparing the estimated $PNEC_{stat}$ obtained (grouping L/E/IC_{50} data by species vs. by phylum), values up to 1.5-fold lower for SiNC, SiNC-DCOIT and SiNC-DCOIT-Ag were estimated for the data grouped by phylum, proposing that this approach may be more conservative at least for these three cases. Concerning the SSDs constructed with NOEC values, it was not possible to perform graphs with data grouped by phylum, since the number of data points would be very low to construct a reliable curve.

On the other hand, comparing the results obtained with the SSDs constructed with L/E/IC_{50} and NOEC data (grouped by species), the calculated $PNEC_{stat}$ values were lower for those using NOEC values, except for DCOIT that was equal and for SiNC-DCOIT-Ag that were slightly higher in the NOEC approach. Thus, considering NOEC instead of L/E/IC_{50} appears to be, as expected, more conservative and advised to use. This magnitude is also considered to be more ecologically relevant and representative for the field situation (Posthuma et al., 2002). Regarding the deterministic approach to calculate PNEC, the use of L/E/IC_{50} produced lower values than the use of NOEC, appearing to be, in this case, a more conservative approach. A very limited number of NOEC values for marine organisms are available in the literature for the tested biocides which can affect the reliability of such based-approach to calculate PNECs.

Regarding the two approaches to calculate the PNEC, it was observed that in this study the deterministic approach provided lower values than the statistical approach, both for L/E/IC_{50} data and for NOEC data, so this seems to be a more conservative approach. However, the deterministic approach only considers the lowest value of L/E/IC_{50} or NOEC, i.e. only one species is considered, while in the statistical approach the PNEC is derived from the HC_5 which in turn was derived considering a set of several species.

With this, although some results different than the expected have been observed, it is possible to state that the technique of encapsulation of biocides in smart ENMs seems to be a promising eco-friendly solution for the development of new materials to be used in anti-fouling coatings, since, globally, the efficacy towards the target species was increased and the hazard for the non-target species reduced. Moreover, this study provides the first holistic hazard assessment for these new materials and that will serve as a basis for future work, and provides additional toxicity data regarding DCOIT and silver. It also includes an extensive compilation of toxicity data for all tested compounds, that can be useful in a regulatory context, but also for industry, as species are ranked by sensitivity, and discrimination between target and non-target can be depicted from the curves, having a protective perspective but looking at the efficacy of the compounds (towards target species).

3.6. References

Adam, N., Schmitt, C., De Bruyn, L., Knapen, D., and Blust, R. (2015). Aquatic acute species sensitivity distributions of ZnO and CuO nanoparticles. *Science of the Total Environment*, 526, 233–242. doi:10.1016/j.scitotenv.2015.04.064.

Aldenberg, T., and Jaworska, J. (2000). Uncertainty of the Hazardous Concentration and Fraction Affected for Normal Species Sensitivity Distributions. *Ecotoxicology and Environmental Safety*, 46(1), 1–18. doi:10.1006/eesa.1999.1869.

Almeida, E., Diamantino, T., and de Sousa, O. (2007). Marine paints: the particular case of antifouling paints. *Progress in Organic Coatings*, 59(1), 2–20. doi:10.1016/j.porgcoat.2007.01.017.

Arrhenius, A., Backhaus, T., Grönvall, F., Junghans, M., Scholze, M., and Blanck, H. (2006). Effects of Three Antifouling Agents on Algal Communities and Algal Reproduction: Mixture Toxicity Studies with TBT, Irgarol, and Sea-Nine. *Archives of Environmental Contamination and Toxicology*, 50(3), 335–345. doi:10.1007/s00244-005-1057-9.

Bellas, J. (2008). Prediction and assessment of mixture toxicity of compounds in antifouling paints using the sea-urchin embryo-larval bioassay. *Aquatic Toxicology*, 88(4), 308–315. doi:10.1016/j.aquatox.2008.05.011.

Bers, A., Dsouza, F., Klijnstra, J., Willemsen, P., and Wahl, M. (2006). Chemical defence in mussels: antifouling effect of crude extracts of the periostracum of the blue mussel *Mytilus edulis*. *Biofouling*, 22(4), 251–259. doi:10.1080/08927010600901112.

Bollmohr, S., Day, J., and Schulz, R. (2007). Temporal variability in particle-associated pesticide exposure in a temporarily open estuary, Western Cape, South Africa. *Chemosphere*, 68(3), 479–488. doi:10.1016/j.chemosphere.2006.12.078.

Botha, T., James, T., and Wepener, V. (2015). Comparative aquatic toxicity of gold nanoparticles and ionic gold using a species sensitivity distribution approach. *Journal of Nanomaterials*, 11. doi:10.1155/2015/986902.

Braithwaite, R., and Fletcher, R. (2005). The toxicity of Irgarol 1051 and Sea-Nine 211 to the non-target macroalga Fucus serratus Linnaeus, with the aid of an image capture and analysis system. *Journal of Experimental Marine Biology and Ecology*, 322(2), 111−121. doi:10.1016/j.jembe.2005.01.015.

Cao, S., Wang, J., Chen, H., and Chen, D. (2010). Progress of marine biofouling and antifouling technologies. *Chinese Science Bulletin*, 56(7), 598–612. doi:10.1007/s11434-010-4158-4.

CCME (2015) Canadian Water Quality Guidelines: Silver. Scientific Criteria Document. Canadian Council of Ministers of the Environment, Winnipeg.

Coll, C., Notter, D., Gottschalk, F., Sun, T., Som, C., and Nowack, B. (2016). Probabilistic environmental risk assessment of five nanomaterials (nano-TiO2, nano-Ag, nano-ZnO, CNT, and fullerenes). *Nanotoxicology*, 10(4), 436–444. doi:10.3109/17435390.2015.1073812.

Dafforn, K., Lewis, J., and Johnston, E. (2011). Antifouling strategies: History and regulation, ecological impacts and mitigation. *Marine Pollution Bulletin*, 62(3), 453–465. doi:10.1016/j.marpolbul.2011.01.012.

DCOIT Assessment Report (2014) Evaluation of active substances under the Regulation (EU) n°528/2012 concerning the making available on the market and use of biocidal products. Norway.

DeForest, D., and Schlekat, C. (2013). Species sensitivity distribution evaluation for chronic nickel toxicity to marine organisms. *Integrated environmental assessment and management*, 9(4), 580–589. doi:10.1002/ieam.1419.

Devilla, R., Brown, M., Donkin, M., Tarran, G., Aiken, J., and Readman, J. (2005). Impact of antifouling booster biocides on single microalgal species and on a natural marine phytoplankton community. *Marine Ecology Progress Series*, 286, 1–12. doi:10.3354/meps286001.

Durán, I., and Beiras, R. (2013). Ecotoxicologically based marine acute water quality criteria for metals intended for protection of coastal areas. *Science of the Total Environment*, 463, 446–453. doi:10.1016/j.scitotenv.2013.05.077.

EU (European Union) (2012). Regulation (EU) No 528/2012 of the european parliament and of the council of 22 may 2012 concerning the making available on the market and use of biocidal products. Official Journal of the European Union.

Fewtrell, L. (2014). Silver: water disinfection and toxicity. Aberystwyth University, Aberystwyth.

Forbes, V. and Callow, P. (2002). Species Sensitivity Distributions Revisited: A Critical Appraisal. *Human and Ecological Risk Assessment: An International Journal*, 8(3), 473–492. doi:10.1080/10807030290879781.

Fusetani, N., and Clare, A. (2006). Antifouling compounds. Berlin: Springer. ISBN: 978-3-540-30016-8.

Garner, K., Suh, S., Lenihan, H., and Keller, A. (2015). Species Sensitivity Distributions for Engineered Nanomaterials. *Environmental Science & Technology*, 49(9), 5753–5759. doi:10.1021/acs.est.5b00081.

Hellio, C., and Yebra, D. (2009). Advances in Marine Antifouling Coatings and Technologies. Boca Raton: CRC Press. ISBN: 9781845696313.

IMO (International Maritime Organization) (2001). International Convention on the Control of Harmful Anti-fouling Systems on Ships.

Jacobson, A., and Willingham, G. (2000). Sea-nine antifoulant: an environmentally acceptable alternative to organotin antifoulants. *Science of The Total Environment*, 258(1-2), 103–110. doi:10.1016/s0048-9697(00)00511-8.

Leung, K., Grist, E., Morley, N., Morritt, D., and Crane, M. (2007). Chronic toxicity of tributyltin to development and reproduction of the European freshwater snail Lymnaea stagnalis (L.). *Chemosphere*, 66(7), 1358–1366. doi:10.1016/j.chemosphere.2006.06.051.

Lützhøf, H., Hartmann, N., Brinch, A., Kjølholt, J., and Baun, A. (2015) Environmental effecs of engineered nanomaterials. Estimation of Predicted No-Effec Concentrations (PNECs). Environmental Protection Agency, Copenhagen.

Macken, A., Byrne, H., and Thomas, K. (2012). Effects of salinity on the toxicity of ionic silver and Ag-PVP nanoparticles to Tisbe battagliai and Ceramium tenuicorne. Ecotoxicology and Environmental Safety, 86, 101–110. doi:10.1016/j.ecoenv.2012.08.025.

Maia, F., Tedim, J., Lisenkov, A., Salak, A., and Zheludkevich, M. (2012) Silica nanocontainers for active corrosion protection. Nanoscale, 4, 1287–1298. doi:10.1016/j.electacta.2012.04.095.

Maia, F. (2015). Micro/nanoreservoirs for controlled release of active species in smart functional coatings. PhD (Doctor of Philosophy) thesis, University of Aveiro, Portugal.

Maia, F., Silva, A., Fernandes, S., Cunha, A., Almeida, A., Tedim, T., Zheludkevich, M., and Ferreira, M. (2015) Incorporation of biocides in nanocapsules for protective coatings used in maritime applications. Chemical Engineering Journal, 270, 150–157. doi: 10.1016/j.cej.2015.01.076.

Maltby, L., Blake, N., Brock, T., and Brink, P. (2005). Insecticide Species Sensitivity Distributions: Importance of Test Species Selection and Relevance To Aquatic Ecosystems. *Environmental Toxicology and Chemistry*, 24(2), 379. doi:10.1897/04-025R.1.

Martins, R., Oliveira, T., Santos, C., Kuznetsova, A., Ferreira, V., Avelelas, F., Caetano, A., Tedim, J., Ferreira, M., Freitas, R., Soares, A., and Loureiro, S. (2017). Effects of a novel

anticorrosion engineered nanomaterial on the bivalve Ruditapes philippinarum. Environmental Science: Nano, 4(5), 1064–1076. doi: 10.1039/C6EN00630B.

Mochida, K., Onduka, T., Amano, H., Ito, M., Ito, K., Tanaka, H., and Fujii, K. (2012). Use of species sensitivity distributions to predict no-effect concentrations of an antifouling biocide, pyridine triphenylborane, for marine organisms. *Marine pollution bulletin*, 64(12), 2807–2814. doi:10.1016/j.marpolbul.2012.09.007.

Mochida, K., Hano, T., Onduka, T., Ichihashi, H., Amano, H., Ito, M., Ito, K., Tanaka, H., and Fujii, K. (2015). Spatial analysis of 4, 5-dichloro-2-n-octyl-4-isothiazolin-3-one (Sea-Nine 211) concentrations and probabilistic risk to marine organisms in Hiroshima Bay, Japan. *Environmental Pollution*, 204, 233–240. doi:10.1016/j.envpol.2015.05.012.

Nguyen, T., Roddick, F., and Fan, L. (2012). Biofouling of water treatment membranes: a review of the underlying causes, monitoring techniques and control measures. Membranes, 2(4), 804–840. doi:10.3390/membranes2040804.

Posthuma, L., Suter II, G., and Traas, T. (2002). Species Sensitivity Distributions in Ecotoxicology. Boca Raton: CRC Press. ISBN: 9781566705783.

Price, A., and Readman, J. (2013). Booster biocide antifoulants: is history repeating itself? Late lessons from early warnings: science, precaution, innvation. Part B–Emerging lessons from ecosystems. *The European Environment Agency. Publications office of the European Union, Luxemburg*, Part 12, 297-310.

Readman, J. (2005). Development, Occurrence and Regulation of Antifouling Paint Biocides: Historical Review and Future Trends *in* Konstantinou I. (2006) Antifouling Paint Biocides (pp. 1–15). Berlin: Springer.

Schipper, C., Smit, M., Kaag, N., and Vethaak, A. (2008). A weight-of-evidence approach to assessing the ecological impact of organotin pollution in Dutch marine and brackish waters; combining risk prognosis and field monitoring using common periwinkles (Littorinalittorea). *Marine Environmental Research*, 66(2), 231–239. doi:10.1016/j.marenvres.2008.02.070.

Salta, M., Wharton, J., Stoodley, P., Dennington, S., Goodes, L., Werwinski, S., Mart, U., Wood, R., and Stokes, K. (2010). Designing biomimetic antifouling surfaces. *Philosophical Transactions of the Royal Society A*, 368(1929), 4729–4754. doi:10.1098/rsta.2010.0195.

Schipper, C., Smit, M., Kaag, N., and Vethaak, A. (2008). A weight-of-evidence approach to assessing the ecological impact of organotin pollution in Dutch marine and brackish waters; combining risk prognosis and field monitoring using common periwinkles (*Littorina littorea*). *Marine Environmental Research*, 66(2), 231–239. doi:10.1016/j.marenvres.2008.02.070.

Shade, W., Hurt, S., Jacobson, A., and Reinert, K. (1993). Ecological Risk Assessment of a Novel Marine Antifoulant. *Environmental Toxicology and Risk Assessment*, 1216, 381–408. doi:10.1520/STP13169S.

Silva, P., Silva, A., Mendo, S., and Loureiro, S. (2014). Toxicity of tributyltin (TBT) to terrestrial organisms and its species sensitivity distribution. *Science of The Total Environment*, 466–467, 1037–1046. doi:10.1016/j.scitotenv.2013.08.002.

Tedim, J., Poznyak, S., Kuznetsova, A., Raps, D., Hack, T., Zheludkevich, M., and Ferreira, M. (2010). Enhancement of Active Corrosion Protection via Combination of Inhibitor-Loaded Nanocontainers. *ACS Applied Materials & Interfaces*, 2(5), 1528–1535. doi: 10.1016/j.electacta.2012.04.095.

TGD (2003) Technical Guidance Document on Risk Assessment — Part II. Institute for Health and Consumer Protection. European Comission Joint Research Centre.

Thomas, K. (2001). The environmental fate and behaviour of antifouling paint booster biocides: A review. *Biofouling*, 17(1), 73–86. doi:10.1080/08927010109378466.

Wendt, I., Backhaus, T., Blanck, H., and Arrhenius, Å. (2016). The toxicity of the three antifouling biocides DCOIT, TPBP and medetomidine to the marine pelagic copepod *Acartia tonsa*. *Ecotoxicology*, 25(5), 871-879. doi:10.1007/s10646-016-1644-8.

Wheeler, J., Grist, E., Leung, K., Morritt, D., and Crane, M. (2002). Species sensitivity distributions: data and model choice. *Marine Pollution Bulletin*, 45(1–12), 192–202. doi:10.1016/S0025-326X(01)00327-7.

Wijnhoven, S., Peijnenburg, W., Herberts, C., Hagens, W., Oomen, A,, Heugens, E., Roszek, B., Bisschops, J., Gosens, I., van de Meent, D., Dekkers, S., De Jong, W., van Zijverden, M., Sips, A., and Geertsma, R. (2009) Nano-silver – a review of available data and knowledge gaps in human and environmental risk assessment. Nanotoxicology 3(2), 109–138. doi:10.1080/17435390902725914.

Williams, T. (2007). The mechanism of action of isothiazolone biocides. *PowerPlant Chemistry*, 9(1), 14.

Willingham, G., and Jacobson, A. (1996). Designing an Environmentally Safe Marine Antifoulant. *ACS Symposium Series*, 640, 224–233. doi:10.1021/bk-1996-0640.ch011.

Yebra, D., Kiil, S., and Dam-Johansen, K. (2004). Antifouling technology – past, present and future steps towards eficiente and environmentally friendly antifouling coatings. *Progress in Organic Coatings*, 50(2), 75–104. doi:10.1016/j.porgcoat.2003.06.001.

Zheludkevich, M., Tedim, J., and Ferreira, M. (2012). “Smart” coatings for active corrosion protection based on multi-functional micro and nanocontainers. Electrochimica Acta, 82, 314–323. doi:10.1016/j.electacta.2012.04.095.

Zhou, F. (2015). Antifouling Surfaces and Materials – From Land to Marine Environment. Berlin: Springer. ISBN: 978-3-662-45204-2.

3.7. Supplementary material

Table 3.1S – Toxicity data (NOEC) for marine organisms exposed to DCOIT or Silver (Ag^+) retrieved from a literature review.

Contaminant	Organism	Species	Endpoint	Value (mg/L)	Reference
DCOIT	Cyanobacteria	*Synechococcus* sp.	72h NOEC	0.001	Devilla et al. (2005)
	Microbial	*Peryphyton community*	72h NOEC	0.050	Arrhenius et al. (2006)
	Microalgae	*Chlorella fusca* var. vacuolata	24h NOEC	0.027	Arrhenius et al. (2006)
		Skeletonema costatum	24h NOEC	0.00048	DCOIT assessment report (2014)
	Macroalgae	*Fucus serratus zygotes*	72h NOEC	0.008	Braithwaite and Fletcher (2005)
	Bivalves	*Mytilus edulis embryo*	48h NOEC	0.207	DCOIT assessment report (2014)
		Crassostrea virginica	48h NOEC	0.010	DCOIT assessment report (2014)
	Echinoderms	*Paracentrotus lividus*	48h NOEC	0.007	Bellas (2008)
Silver	Crustaceans	*Tisbe batagliai*	24h NOEC	0.032	Macken et al. (2012)
	Crustaceans	*Tisbe batagliai*	48h NOEC	0.010	Macken et al. (2012)
	Fish	*Oncorhynchus mykiss*	96h NOEC	0.108	EPA ecotox database (cons. 20.06.17)

Table 3.2S – Compilation of HC_5 and PNEC values (mg/L) for different biocides and nanomaterials obtained from SSD curves constructed with different species toxicity data. n.i. – no information available. n.i. – no information available; St Chronic – short-term chronic.

	Compound	Unit	Parameter	Compartment	HC_5	$PNEC_{stat}$	$PNEC_{determ}$	Reference
Biocides	DCOIT	mg DCOIT/L	Acute/St Chronic L/E/IC_{50}	marine	0.001	0.0002	0.0000004	This study
		mg DCOIT/L	Acute/St Chronic NOEC	marine	0.001	0.0002	0.0000048	This study
	PTPB	mg DCOIT/L	Acute L/E/IC_{50}	marine	0.0005	-	0.0000004	Mochida et al. (2015)
		mg PTPB/L	Acute L/E/IC_{50}	marine	0.0008	-	0.00003	Mochida et al. (2012)
	TBTO	mg TBT/L	Chronic L/NOECs	marine	0.000004	-	-	Leung et al. (2007)
		mg TBT/L	Chronic L/NOECs	freshwater	0.00003	-	-	Leung et al. (2007)
	Silver	mg Ag^+/L	Acute/St Chronic L/E/IC_{50}	marine	0.009	0.002	0.000006	This study
		mg Ag^+/L	Acute/St Chronic NOEC	marine	0.007	0.004	0.00007	This study
		mg Ag^+/L	Acute L/E/IC_{50}	marine	0.0005	-	-	CCME (2015)
		mg Ag^+/L	Acute L/E/IC_{50}	freshwater	0.008	-	-	CCME (2015)
Nanomaterials	SiNC	mg SiNC/L	Acute/St Chronic L/E/IC_{50}	marine	0.101	0.020	0.000001	This study
		mg SiNC/L	Acute/St Chronic NOEC	marine	0.044	0.009	0.0001	This study
	SiNC-DCOIT	mg DCOIT/L	Acute/St Chronic L/E/IC_{50}	marine	0.023	0.005	0.000007	This study
		mg SiNC/L	Acute/St Chronic L/E/IC_{50}	marine	0.100	0.020	0.000029	This study
		mg SiNC-DCOIT/L	Acute/St Chronic L/E/IC_{50}	marine	0.123	0.025	0.000036	This study
		mg DCOIT/L	Acute/St Chronic NOEC	marine	0.001	0.0002	0.00001	This study
		mg SiNC/L	Acute/St Chronic NOEC	marine	0.008	0.002	0.00004	This study
		mg SiNC-DCOIT/L	Acute/St Chronic NOEC	marine	0.009	0.002	0.0001	This study
	SiNC-Ag	mg Ag^+/L	Acute/St Chronic L/E/IC_{50}	marine	0.024	0.005	0.000017	This study
		mg SiNC/L	Acute/St Chronic L/E/IC_{50}	marine	0.144	0.029	0.000109	This study
		mg SiNC-Ag/L	Acute/St Chronic L/E/IC_{50}	marine	0.187	0.037	0.0001	This study
		mg Ag^+/L	Acute/St Chronic NOEC	marine	0.015	0.003	0.00012	This study
		mg SiNC/L	Acute/St Chronic NOEC	marine	0.089	0.018	0.0007	This study
		mg SiNC-Ag/L	Acute/St Chronic NOEC	marine	0.114	0.023	0.001	This study
	SiNC-DCOIT-Ag	mg DCOIT/L	Acute/St Chronic L/E/IC_{50}	marine	0.009	0.002	0.000002	This study
		mg Ag^+/L	Acute/St Chronic L/E/IC_{50}	marine	0.009	0.002	0.000002	This study

	mg SiNC/L	Acute/St Chronic L/E/IC_{50}	marine	0.044	0.009	0.00001	This study
	mg SiNC-DCOIT-Ag	Acute/St Chronic L/E/IC_{50}	marine	0.061	0.012	0.00001	This study
	mg DCOIT/L	Acute/St Chronic NOEC	marine	0.010	0.002	0.00007	This study
	mg Ag^{+}/L	Acute/St Chronic NOEC	marine	0.010	0.002	0.00007	This study
	mg SiNC/L	Acute/St Chronic NOEC	marine	0.045	0.009	0.00032	This study
	mg SiNC-DCOIT-Ag	Acute/St Chronic NOEC	marine	0.071	0.014	0.005	This study
MWCNT	n.i.	n.i.	marine	-	-	0.043	Lützhøf et al. (2015)
CB	n.i.	n.i.	marine	-	-	5	Lützhøf et al. (2015)
nano-CuO	mg Cu/L	Acute L/EC_{50}	freshwater	0.15	-	-	Adam et al. (2015)
		Acute LC_{50}	freshwater	-	-	0.00034	Lützhøf et al. (2015)
nano-Au	mg Au/L	Acute L/E/C_{50}	freshwater	42.78	-	-	Botha et al. (2015)
nano-Ag	mg Ag/L	Acute/Chronic NOEC	freshwater	0.00002	0.00002	-	Coll et al. (2016)
		Acute EC_{50}	freshwater	-	-	0.000012	Lützhøf et al. (2015)
nano-TiO2	mg Ti/L	Acute/Chronic NOEC	freshwater	0.016	0.016	-	Coll et al. (2016)
	mg Ti/L	St Chronic NOEC	freshwater	-	-	0.018	Lützhøf et al. (2015)
nano-ZnO	mg Zn/L	Acute L/EC_{50}	freshwater	0.06	-	-	Adam et al. (2015)
	mg Zn/L	Acute/Chronic NOEC	freshwater	0.001	0.001	-	Coll et al. (2016)
	mg Zn/L	St Chronic NOEC	freshwater	-	-	0.0025	Lützhøf et al. (2015)
Carbon nanotubes	n.i.	Acute/Chronic NOEC	freshwater	0.056	0.056	-	Coll et al. (2016)
	n.i.	St Chronic NOEC	freshwater	-	-	0.00084	Lützhøf et al. (2015)
Fullerenes	n.i.	Acute/Chronic NOEC	freshwater	0.004	0.004	-	Coll et al. (2016)
nano-nZVI	n.i.	Acute LOEC	freshwater	-	-	0.005	Lützhøf et al. (2015)
Nano-CeO_2	n.i.	Acute LC_{50}	freshwater	-	-	0.0052	Lützhøf et al. (2015)

Chapter IV

General discussion and final considerations

4. General discussion and final considerations

Biofouling is a worldwide problem that affects all submerged structures and that can lead to several negative environmental and socio-economic impacts (Jacobson and Willingham, 2000; Yebra et al., 2004; Gama et al., 2009; Hellio and Yebra, 2009; Cao et al., 2010). In order to prevent biological incrustation, different techniques have been applied over the years, particularly the use of coatings containing biocides. One of the most widely used biocide in the world was TBT, which was banned in 2008 due to its toxic effects on non-target species at very low concentrations, bioaccumulation and persistence in environment (IMO, 2001; Yebra et al., 2004; Readman, 2005; Hellio and Yebra, 2009). It was then necessary to develop new biocides of equal efficacy but eco-friendlier and booster biocides, like DCOIT, emerged (Thomas, 2001; Dafford et al., 2011; Zhou, 2015). DCOIT, although less toxic than TBT, degrades in seawater in less than 24 h (Shade et al., 1993; Willingham and Jacobson, 1996; Thomas, 2001), still represents a concern since it has a broad action spectrum and causes detrimental effects on target and non-target species in the first hours of exposure (Jacobson and Willingham, 2000; Yebra et al., 2004; Dafforn et al., 2011; Price and Readman, 2013). Recently, to reduce the toxicity of biocides, an innovative solution has been developed using ENMs, like SiNC, to encapsulate biocides in order to control their leaching rate to environment (Tedim et al., 2010; Maia et al., 2012; Zheludkevich et al., 2012; Maia et al., 2015; Avelelas et al., 2017). Three examples of nanomaterials produced using this technique are SiNC loaded with DCOIT (SiNC-DCOIT), SiNC coated with silver nitrate (SiNC-Ag) and a combined nanomaterial with the two biocides (SiNC-DCOIT-Ag). Although some information on the encapsulation of biocides already exists in literature for zinc and copper pyrithiones (Zn-PT and Cu-PT) (Avelelas et al., 2017) few is known about the encapsulation of DCOIT and silver in silica nanocapsules, excepting some information regarding SiNC-DCOIT efficacy, assessed using the bacteria *Vibrio fischeri* (Maia et al., 2015).

Globally, SiNC was the less toxic compound while DCOIT and Ag^{+} were very toxic or even extremely toxic to target and non-target species. The encapsulation of the two biocides in the silica nanocontainers reduced their toxicity for pratically all non-target organisms. Regarding target species (*V. fischeri*, *P. tricornutum* and *M. galloprovincialis*), it was also observed a reduction of toxicity for SiNC-DCOIT to the three target species, SiNC-Ag to the diatom and bivalve and SiNC-DCOIT-Ag only towards *M. galloprovincialis*. Despite this reduction, the biocides remained very toxic or extremely toxic to the target species when loaded into SiNC, indicating that encapsulation does not

seem to reduce their anti-fouling efficacy. Ideally, encapsulation should reduce the toxicity of biocides to non-target species while increasing, or at least maintain, the effect against the target species. Therefore, this tecnhique seems to be a promising solution for combating/retarding biofouling, particularly if used at low concentrations combined with other techniques (e.g. the same nanomaterials loaded with different biocides, other nanomaterials loaded with the same biocides, free biocides at very low concentrations) to achieve a broader action spectrum with low environmental impact.

On the other hand, although SiNC was the less toxic compound, it was still classified as toxic for several species, which was not expected. The first hypothesis raised to justify this was related to the use of the surfactant CTAB in the production process of the nanocapsules. As seen in the toxicity tests results, CTAB is very toxic to the tested organisms at low concentrations. Although this compound was not detected after its elimination process, it can be possibly that it continues present or with some other residual compounds that can be responsible for the SiNC toxicity.

The results obtained using SSDs support these previous findings, since the obtained HC_5 and PNEC values were higher for the encapsulated biocides comparing with their isolated form, both using $L/E/IC_{50}$ and NOEC data. This indicates that the encapsulation of DCOIT and silver not only decreases their toxicity but also reduces the hazard of these biocides to a set of marine species of different trophic levels. Moreover, the silica nanocapsules are trapped in the coatings matrix and are hardly released, reducing the possibility of being carried by the currents and induce effects on organisms that are not involved in the biofouling process.

Shortly, the main conclusions that arose from the present study are that, globally, SiNC-DCOIT-Ag can be considered the best biocidal product, with better efficacy than SiNC-DCOIT and SiNC-Ag and even than free DCOIT and free Ag^+. However, SiNC-DCOIT-Ag has some issues regarding toxicity to non-target species, since it was still toxic towards the tested organisms and the one representing more hazard to the environment (despite less toxic and hazardous than the free biocides). On the other hand, empty SiNC and SiNC-Ag were the nanomaterials representing less hazard to environment. Although less toxic against non-target species, these materials proved to have lower anti-fouling efficacy than SiNC-DCOIT and SiNC-DCOIT-Ag.

The present work contributed to complement literature on the toxicity of DCOIT and silver and, especially, to provide information on the toxicity of the compounds SiNC-DCOIT, SiNC-Ag and SiNC-DCOIT-Ag which did not yet exist. Besides that, the use of SSDs allowed to complement the information on the effects of these novel nanomaterials, providing an environmental hazard assessment of the tested compounds and enabling to have a more general and accurate picture of their impacts in the

environment, which is of extreme importance in the case of compounds for which few to no information is available.

Thus, this work can serve as basis for future studies, such as biomarkers, genotoxicity and chronic toxicity testing of these compounds and bioaccummulation tests, which is an important endpoint also requested by EU regulations. To complement the already obtained information more acute tests with other species may be performed, especially with organisms at higher trophic levels, such as the fishes (e.g. a couple of preliminary tests have already been carried out using *Solea senegalensis*).

4.1. References

Avelelas, F., Martins, R., Oliveira, T., Maia, F., Malheiro, E., Soares, A., Loureiro, S., and Tedim, J. (2017). Efficacy and Ecotoxicity of Novel Anti-Fouling Nanomaterials in Target and Non-Target Marine Species. Marine Biotechnology, 19(2), 164–174. doi:10.1007/s10126-017-9740-1.

Cao, S., Wang, J., Chen, H., and Chen, D. (2010). Progress of marine biofouling and antifouling technologies. *Chinese Science Bulletin*, 56(7), 598–612. doi:10.1007/s11434-010-4158-4.

Dafforn, K., Lewis, J., and Johnston, E. (2011). Antifouling strategies: History and regulation, ecological impacts and mitigation. *Marine Pollution Bulletin*, 62(3), 453–465. doi:10.1016/j.marpolbul.2011.01.012.

Gama, B., Pereira, R., and Coutinho, R. (2009). Bioincrustação marinha *in* Pereira, R., Soares-Gomes, A. (2009). Biologia Marinha (2º ed). Rio de Janeiro: Interciência, pp. 299–318.

Hellio, C., and Yebra, D. (2009). Advances in Marine Antifouling Coatings and Technologies. Boca Raton: CRC Press. ISBN: 978-1-84569-631-3.

IMO (International Maritime Organization) (2001). International Convention on the Control of Harmful Anti-fouling Systems on Ships.

Jacobson, A., and Willingham, G. (2000). Sea-nine antifoulant: an environmentally acceptable alternative to organotin antifoulants. *Science of The Total Environment*, 258(1-2), 103–110. doi:10.1016/s0048-9697(00)00511-8.

Maia, F., Tedim, J., Lisenkov, A., Salak, A., and Zheludkevich, M. (2012) Silica nanocontainers for active corrosion protection. *Nanoscale*, 4, 1287–1298. doi:10.1016/j.electacta.2012.04.095.

Maia, F., Silva, A., Fernandes, S., Cunha, A., Almeida, A., Tedim, T., Zheludkevich, M., and Ferreira, M. (2015) Incorporation of biocides in nanocapsules for protective coatings used in maritime applications. Chemical Engineering Journal, 270, 150–157. doi: 10.1016/j.cej.2015.01.076.

Price, A., and Readman, J. (2013). Booster biocide antifoulants: is history repeating itself? Late lessons from early warnings: science, precaution, innvation. Part B–Emerging lessons from ecosystems. *The European Environment Agency. Publications office of the European Union, Luxemburg*, Part 12, 297-310.

Readman, J. (2005). Development, Occurrence and Regulation of Antifouling Paint Biocides: Historical Review and Future Trends. *The Handbook of Environmental Chemistry Antifouling Paint Biocides*, 5, 1–15. doi:10.1007/698_5_047.

Shade, W., Hurt, S., Jacobson, A., and Reinert, K. (1993). Ecological Risk Assessment of a Novel Marine Antifoulant. *Environmental Toxicology and Risk Assessment*, 1216, 381–408.

Tedim, J., Poznyak, S., Kuznetsova, A., Raps, D., Hack, T., Zheludkevich, M., and Ferreira, M. (2010). Enhancement of Active Corrosion Protection via Combination of Inhibitor-Loaded Nanocontainers. *ACS Applied Materials & Interfaces*, 2(5), 1528–1535. doi: 10.1016/j.electacta.2012.04.095.

Thomas, K. (2001). The environmental fate and behaviour of antifouling paint booster biocides: A review. *Biofouling*, 17(1), 73–86. doi:10.1080/08927010109378466.

Willingham, G., and Jacobson, A. (1996). Designing an Environmentally Safe Marine Antifoulant. *ACS Symposium Series*, 640, 224–233. doi:10.1021/bk-1996-0640.ch011.

Yebra, D., Kiil, S., and Dam-Johansen, K. (2004). Antifouling technology – past, present and future steps towards eficiente and environmentally friendly antifouling coatings. *Progress in Organic Coatings*, 50(2), 75–104. doi:10.1016/j.porgcoat.2003.06.001.

Zheludkevich, M., Tedim, J., and Ferreira, M. (2012). “Smart” coatings for active corrosion protection based on multi-functional micro and nanocontainers. Electrochimica Acta, 82, 314–323. doi:10.1016/j.electacta.2012.04.095.

Zhou, F. (2015). Antifouling Surfaces and Materials – From Land to Marine Environment. Berlin: Springer. ISBN: 978-3-662-45204-2.